NATURAL COMPOUNDS ISOLATED FROM WILD ALASKA BOG BLUEBERRIES INTERVENE WITH MOLECULAR TARGETS OF NEUROINFLAMMATION

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A

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Abstract

NADPH Oxidase (NOX) has emerged as a key mediator of inflammatory processes that are prevalent in acute and chronic pathologies of the nervous system, cardiovascular system, and the immune system. Activation of NOX results in the formation of superoxide, a specific type of reactive oxygen species (ROS). Excessive accumulation of superoxide causes severe oxidative stress and ultimately, progressive cellular damage and degeneration. Despite the implications of NOX in a multitude of pathologies, pharmaceutical interventions against this molecular target remain non-existent.

A diet rich in fruits and vegetables has immense health benefits beyond the high content of antioxidant compounds. Dietary intake of blueberries improves age related cognitive deficits and alleviates inflammatory damage as shown through human trials and animal studies. These findings imply that blueberries harbor specific inhibitors against molecular targets implicated in neuronal inflammation. Our investigations unveil natural compounds present in wild Alaska bog blueberries that potently inhibit NOX activity, reduce oxidative stress, and protect neuronal health in a cellular model of neuroinflammation. These studies illuminate nutrition-guided strategies as potential therapies for the prevention and intervention of neurodegeneration and cognitive decline associated with aging and with disease.

Dedication

I am honored to dedicate this thesis to the two most important people in my life. Their support, encouragement, and friendship have guided me to discover the tools I need to succeed in this world. These individuals have maintained confident in my abilities even when I have not. They taught me to always do what feels good in my heart and their mentorship has instilled in me the value of education as well as the perseverance to work hard for what I want. To the two that have helped make my dreams my reality, to my parents - this is for you.

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List of Abbreviations

ABBX	Aqueous blueberry extract
ALS	Amyotrophic lateral sclerosis
BBX	Blueberry extract
CGD	Chronic granulomatous disease
CNS	Central nervous system
Cyt b_{558}	Flavocytochrome b_{558}
DA	Dopaminergic
DRM	Detergent resistant membrane
DSM	Detergent soluble membrane
FB	Fairbanks
FITC	Fluorescein isothiocyanate
FX	Fox
H_2DCFDA	2',7'-dihydrodicholorfluorecein diactate
HRP	Horseradish peroxidase
IH	Intermittent hypoxia
LM	Lake Minchumina
LR	Lipid raft
MβCD	Methyl-β-cyclodextrin
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	N-acetylcystein

NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH Oxidase
NP_{BB}	Non-polar blueberry extract
nSMase	Neutral sphingomyelinase
OBBX	Organic blueberry extract
ORAC	Oxygen radical absorbance capacity
PD	Parkinson disease
PKC	Protein kinase C
PMA	Phorbol 12-myristate-13-acetate
PO_{BB}	Polar blueberry extract
ROS	Reactive oxygen species
TNFα	Tumor necrosis factor alpha
UA	Ursolic acid

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Chapter 1

NADPH Oxidase As A Forgotten Therapeutic Target: The Pursuit Of Free Radical Regulation*

1.1 Abstract

Inflammatory processes and severe oxidative stress marked by increased reactive oxygen species (ROS), are both hallmarks of chronic pathologies of the central nervous system (CNS), acute CNS trauma, several psychiatric disorders, and also general aging. Inflammation and oxidative stress both largely contribute to neuronal degeneration and cognitive decline. Recent findings attribute a vital role to members of the NADPH oxidase (NOX) family in the onset and progression of age-related diseases and also general aging. NOX enzymes harbor a unique capacity to deliberately generate the specific ROS known as superoxide. NOX activity depends on a complex assembly of membrane and cytosolic subunits in lipid rafts, dynamic microdomains of the cellular plasma membrane. Diets rich in berry fruit, particularly blueberries, protect neurons from oxidative stress and improve cognitive function with health benefits far beyond their high antioxidant capacity. The research presented in this thesis demonstrates that natural compounds present in wild Alaska bog blueberries potently and specifically intervene with NOX activity through lipid raft modulation. These findings illuminate how regulation of NOX function impacts neuronal degeneration and provides a new concept of therapeutic intervention as well as strategies for prevention.

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1.2 Introduction

Oxidative stress results from a disruption of cellular redox homeostasis and is characterized either by an overabundance of reactive oxygen species (ROS), a lack of antioxidant defense, or a combination of both. Only recently, the family of NADPH Oxidase (NOX) enzymes that deliberately generate the ROS known as superoxide, have emerged as key players in a plethora of pathologies (Lambeth et al. 2008). NOX-dependent oxidative stress in conjunction with inflammatory stress is particularly prevalent in acute central nervous system (CNS) injuries, many chronic CNS diseases, some psychiatric disorders, and also in general aging. With respect to the CNS diseases, NOX activity is frequently associated with chronic and acute neurodegenerative conditions such as Alzheimer's, Parkinson's, Neimann-Pick disease, Huntington's, Amyotrophic lateral sclerosis (ALS), mild cognitive impairments, trauma, and also with stroke and ischemia. NOX enzymes are expressed in a cell and tissue specific fashion and are prevalent in neurons of the central and peripheral nervous systems as well as microglia (Lambeth, 2002; Block et al. 2007). These multi-subunit enzymes are composed of two plasma membrane subunits, at least three cytosolic subunits, and functionally assemble within lipid rafts (LR) domains of the cellular plasma membrane. LR domains are highly dynamic microdomains that harbor unique compositions of lipids and proteins (Sorescu et al. 2002). Our understanding regarding the free radical regulation of NOX both in physiology and pathology is marginal and current pharmacology is poor with respect to specificity, potency, and toxicity. Therefore, the development of potent NOX inhibitors would be beneficial in decelerating, containing, or

preventing the progression of NOX-dependent pathologies. Health benefits of nutrition are long known but there is still a significant lack of molecular understanding. Berry fruit, in particular blueberries, interfere with inflammatory and oxidative stress, prevent cognitive decline in diseases and aging, and abolish or even reverse cognitive deficits (Goyarzu et al. 2004; Joseph et al. 2006; Duffy et al. 2008, Krikorian et al. 2010). These findings imply that berry fruit might contain specific natural compounds with the capacity to potently inhibit NOX aside from their high antioxidant potential. This research represents a natural products-guided drug discovery endeavor to isolated potent NOX inhibitors with low cellular toxicity.

1.3 NADPH Oxidase in Disease and Aging

The NADPH oxidase (NOX) enzyme is a molecular target of neuroinflammation and serves a pivotal role in the onset and progression of many inflammatory conditions. NOX has been related to innate immune disorders such as Chronic granulomatous disease (CGD) as well as thyroid metabolic disorders, cardiovascular disease, and to a plethora of neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's, Parkinson's, and also to general aging. This wide range of NOX-associated conditions results from the presence of five distinct NOX isoforms, an intricate tissue-specific expression pattern of these isoforms, and distinct regulation of the isoforms. A wonderful review by Bedard and Kruase (2007) extensively details on the participation of NOX in individual diseases. Here we trace a brief overview of some of the immune, thyroid, cardiovascular, and CNS diseases in which NOX involvement is evident.

1.3.1 Immune

Chronic granulomatous disease (CGD) is a hereditary immune disorder transmitted as either a deletion, frame-shift, nonsense, or missense mutation on the X chromosome encoding region of the glycoprotein $gp91^{phox}$ (Heyworth et al. 2003; Lewis et al. 2009). Inactivation of the small membrane NOX subunit, $p22^{phox}$, also results in a CGD phenotype and is transmitted in an autosomal recessive fashion (Heyworth et al. 2003). Granulomata tumors are a result of CGD and are composed of granulation tissue produced in response to chronic infection or inflammation. Abnormalities in either $p22^{phox}$ or $gp91^{phox}$ result in the inability of phagocytes to produce a burst of ROS upon stimulation. This excessive ROS formation is an important contributor to the process of phagocytosis in which phagocytes such as macrophages, neutrophils, and monocytes destroy bacteria or other apoptotic cellular debris (Silva, 2010). One of the rarest forms of CGD is caused by mutations in cytochrome b-245 (CYBA), which encodes the $p22^{phox}$ subunit of phagocyte NOX and only leads to defective intracellular killing yet not affecting phagocytosis (Teimourian et al. 2008). One study using the nmf333 mouse strain, an animal model of $p22^{phox}$ deficiency, revealed that the deletion of this NOX membrane subunit exhibited a phenotype consisting of CGD-like immune defects (Nakano et al. 2008).

1.3.2 Thyroid

The thyroid gland carries out a peroxidase-dependent iodination of thyroglobulin, a key step in the biosynthesis of thyroid hormones in a reaction that requires NOX

generated H_2O_2 (Dupuy et al. 1999). The thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) are produced by the thryroid gland and are responsible for the regulation of metabolism. Two NOX isoenzymes, DUOX1 and DUOX2, expressed in thyroid have been identified by molecular cloning and generate the H_2O_2 utilized by thyroperoxidase (De Deken et al. 2000). The generated H_2O_2 acts as an electron acceptor for iodide oxidation and covalently links oxidized iodide to tyrosine residues in thyroglobulin as the rate limiting step in thyroid hormone production (Rigutto et al. 2009). The DUOX2 gene is known to be essential for thyroid hormone biosynthesis and has be irrefutably demonstrated with mutations and genetic variants to have implications in hypothyroidism (Vigone et al. 2005).

1.3.3 Cardiovascular

Vascular ailments such as hypertension and hypercholesterolemia can lead to heart and blood vessel damage that sets the stage for stroke, atherosclerosis, heart failure, and myocardial infarction. NOX1 and NOX2 are expressed in cardiovascular cells and their activities are acutely increased by pathophysiological stimuli similar to the NOX response in neutrophils (Dworakowski et al. 2006). NOX1 over-expression in transgenic hypertensive rats resulted in a marked increase in systolic blood pressure and hypertrophy in response to stimuli. In contrast, NOX1 knockout mice exhibit a lowering of basal blood pressure and complete protection against stimulus-induced increases in blood pressure and medial hypertrophy (Gavazzi et al. 2006; Matsuno et al. 2005; Wingler et al. 2001). Oxidative stress has been recognized as an important contributor to hypertensive

disease and the redox signaling regulated by NOX generated ROS has been linked to the pathophysiology of several cardiovascular diseases (Lambeth, 2007). Evidence points to a critical role of NOX in vascular remodeling associated with hypertension, although a more thorough understanding of NOX regulation and the mechanisms through which the enzyme modulates redox signaling is necessary for the development of new therapeutic interventions for hypertensive cardiovascular conditions.

1.3.4 Central Nervous System

The NOX2 isoform is found predominantly in the CNS (Bedard and Krause, 2007). NOX plays an important role in various CNS pathologies involving cortical and diencephalic structures including Alzheimer disease, Parkinson's disease, ALS , other forms of dementia, and mild cognitive impairment. Neuronal damage associated with Alzheimer's disease results in the progressive impairment of memory and cognitive decline concurrent with the pro-inflammatory Amyloid-β (AB) protein expression. AB expression can directly affect neurons and can cause neuronal damage via microglia release of neurotoxic factors such as NO, TNFα, and superoxide (Block et al. 2007). Elimination of AB-induced oxidative damage through inhibition of microglial NOX serves as an attractive therapeutic target for treatment of Alzheimer's disease (Wilkinson and Landreth, 2006).

Parkinson disease (PD) is a degenerative disease of the CNS characterized by the loss of dopaminergic (DA) neurons and the presence of cytoplasmic inclusions known as Lewy bodies. Post-mortem studies on PD have indicated oxidative damage resulting from

NOX2-mediated ROS. Recent research shows elevated NOX2 expression in activated microglia of mice injected with 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin known to induce permanent PD-like symptoms by targeting DA neurons (Wu et al. 2003). The abundance of activated microglia in patients with Parkinson's disease suggests NOX as a crucial target in the development of new therapeutics for the prevention and treatment of this CNS disease.

Amyotrophic lateral sclerosis (ALS) is characterized by NOX-generated superoxide and the loss of motor neurons in the motor nuclei of the brain stem (Harraz et al. 2008). The resulting inflammation and cell loss causes progressive paralysis and ultimately leads to death. While the majority of ALS cases appear independent, familial cases are related to mutations in the superoxide dismutase-1 (SOD1) gene. This gene codes the SOD1 catabolic enzyme, which converts superoxide ($O_2^{\bullet-}$) to H_2O_2 (Crow et al. 2005). Mutations in this gene inhibit the GTPase Rac1 and result in the inability to convert NOX mediated superoxide to H_2O_2 both of which elevate redox stress in motor neurons and increase neuroinflammation leading to CNS disease progression (Harraz et al. 2008; Crow et al. 2005). SOD1 mimetics have been administered at disease onset in ALS mice and are reported to slow disease progression (Crow et al. 2005); however a better understanding of NOX inhibition may increase therapeutic promise for this condition.

1.4 NADPH Oxidase Implications in Aging

Free radicals predominantly generated by NOX and mitochondria play an important role in aging as well as the pathogenesis of age-related diseases. An imbalance between ROS generation and cellular antioxidant capacity leads to oxidative stress and ultimately damage to various tissues. Major antioxidant defense systems rest on enzymatic and nonenzymatic activities including reduced glutathione (GSH), superoxide dismutase (SOD), catalase, glutathione peroxidase, and heme oxygenase (Guo et al. 2007). While oxidative damage can be observed in mitochondria during aging, antioxidants have shown to slow the ageing process by targeting mitochondrial ROS. Reduction of neurological deficits in aged animal models have been implicated through anti-aging mechanisms, such as free radical scavenging, and by incorporating foods rich in flavonoids, epicatechins, and anthocyanins such as spinach, strawberries, and blueberries (Cao et al. 1999; Gemma et al. 2002). Reducing NOX-generated superoxide through inhibition, rather than through an antioxidant defense system, may also serve as a target for correcting this imbalance.

1.5 Microglia: A Key Target For Neuroinflammation

Microglia compose approximately fifteen percent of neuroglia in the brain and their principal role is to identify neurotoxic stimuli and protect the CNS from permanent neuronal damage. Under healthy physiological conditions, microglia are involved in various aspects of brain development as well as neuronal survival by releasing trophic and anti-inflammatory factors (Block et al. 2007). On the other hand, over-stimulation of

microglia triggers the release of massive amounts of ROS, nitric oxide (NO), and cytokines that exacerbate and progressively spread inflammatory processes in the CNS. Micrglia-dervied ROS enhance the formation of other potent free radicals such a peroxynitrite, causing further damage to proteins, lipids, and DNA all of which lead to neuronal cell death (Wilkinson and Landreth, 2006). A number of studies attribute microglia neurotoxicity to NOX-mediated release of superoxide (Qin et al. 2005). Recent research shows that mice injected with 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin known to induce permanent neurodegenerative damage, over-express NOX after induced microglia activation (Wu et al. 2002). Neurodegenerative diseases in general have long been associated with microglia over-activation as a consequence of oxidative stress and neuroinflammation. The ability to inhibit free radical formation associated with microglia activation, without disrupting their normal function, is critical to decreasing or preventing disease progression.

1.6 NADPH Oxidase Isoforms

While the structure and function of NOX isoforms are somewhat conserved, their signaling pathways, activation, and inhibition mechanisms may vary depending on their presence and role in various cell types. The study of this family of proteins may clarify novel physiological mechanisms for free radical regulation in various NOX related diseases. The family of NOX enzymes consists of seven isoforms: NOX1-NOX5, DUOX1, DUOX2, and is defined by the large membrane subunit of the phagocytic NADPH oxidase, $gp91^{phox}$ (NOX2). These isoforms are expressed in various mammalian

tissues and have been identified in a plethora of cell types including hematopoietic stem cells, endothelial cells, epithelial cells, myocardial cells, muscle cells, hepatocytes, and neurons (Bedard and Krause, 2007).

1.6.1 NOX1-NOX5

The best-characterized NOX isoform is NOX2. NOX2 is expressed in phagocytic leukocytes and involved in leukocyte respiratory burst through activation by invading pathogens (Bokoch and Knaus, 2003). Sharing structural homology of the gp91phox membrane subunit, other NOX isoforms differ significantly from NOX2 in their subunit composition and regulation and in turn many of the regulatory mechanisms for NOX isoforms are still inadequately defined (Bokoch and Knaus, 2003). The regulation of NOX2 is orchestrated by three cytosolic subunits (p47phox, p67phox, p40phox) and the ancillary factor Rac2/1, which all translocate to the plasma membrane and form an intricate network of protein interactions with the membrane bound subunits of NOX2 (Lambeth et al. 2007). Posttranslational modification of cytosolic subunits is necessary for proper translocation and protein interactions. The search for homologues of the NOX2 regulatory proteins $p47^{phox}$ and $p67^{phox}$ led to the identification of NOXO1 ($p47^{phox}$ homologue) and NOXA1 ($p67^{phox}$ homologue) (Banfi et al. 2003). These two homologous proteins, along with Rac1 (Lambeth et al. 2007), serve as the regulatory activators of NOX1 which is highly expressed in colon epithelium (Gianni et al. 2008). NOX3 requires the NOXO1 regulatory subunit and is expressed in embryonic kidney cells and in the inner ear (Cheng et al. 2001). More specifically NOX3 has been identified in the

cochlea and vestibular sensory epithelium (Lambeth, 2007). NOX4 expression is observed in renal distal tubules in the kidney cortex and its regulatory constituents remain unknown (Geiszt et al. 2000; Shiose et al. 2001). NOX5 is expressed primarily in testis and spleen with weak expression in ovary, placenta, and pancreas; significantly different from other NOX isoforms, NOX5 does not require $p22^{phox}$ for superoxide production. The regulation of NOX5 is dependent on the synergism of calcium-dependent and phosphorylation-dependent pathways and its structure differs from that of NOX1-4 in that it contains four calcium-binding EF-hand motifs (Lambeth, 2007) and has an additional membrane spanning alpha helix that may serve a similar function to that of $p22^{phox}$ (Cheng et al. 2001).

1.6.2 Dual Oxidases

The dual oxidases (DUOX1 and DUOX2) are NOX isoforms that exclusively generate H_2O_2 in thyroid and human bronchial epithelial cells (De Deken et al. 2002). Similar to NOX5, the DOUX enzymes are regulated by calcium and are $p22^{phox}$ independent (Lambeth et al. 2007). In addition to a catalytic core structure equivalent to the superoxide-producing NOX isoforms 1-5, the DUOX structure contains an extended extracellular peroxidase-like domain at the NH_2-terminal followed by a membrane-spanning segment and an intracellular domain including two calcium binding motifs (De Deken et al. 2000). In contrast to NOX, DUOX are not associated with cytosolic factors but rather undergo a maturation process for activation. The non-functional, improperly glycosylated form of DUOX is maintained in the endoplasmic reticulum where it

associates with DUOX maturation factors, DUOXA1 and DUOXA2. These maturation factors assist in the proper folding of DUOX enzymes and in the exit of DUOX from the endoplasmic reticulum hence DUOX expression at the apical cell surface (Rigutto et al. 2009).

1.7 NADPH Oxidase Structure and Function

NOX isoforms 1-5 are multimeric enzymes consisting of multiple hetero-subunits whose associations commence in a stimulus-dependent manner to form a functional O_2^- producing complex (Figure 1). Dependent on the localization of the isoform, variations may occur in the membrane and cytosolic components of the enzyme, their associations, and the enzymes overall regulation. The most thoroughly understood isoform, NOX2, serves as a homology model for the other NOX isoforms in attempt to better understand their regulation. In this review the structure and functional subunit assembly of NOX2 is described in detail to model the fashion in which all NOX isoforms may generate superoxide.

1.7.1 Membrane Bound Subunits

NOX2 consist of two specific integral membrane proteins, $gp91^{phox}$ (β subunit) and $p22^{phox}$ (α subunit) comprised of 570 and 195 amino acids, respectively (Bedard and Krause, 2007). These subunits associate in a 1:1 manner forming the inactive catalytic core of the NOX enzyme, otherwise known as flavocytochrome b_{558} (cyt b_{558}) (Lin et al. 2007). In $gp91^{phox}$, 300 N-terminal amino acids compose six trans-membrane α-helices

and the cytosolic C-terminus includes binding sites for both flavin adenine dinucleotide (FAD) and NADPH (Figure 2). $Gp22^{phox}$ is situated in the membrane with three α-helical domains and also includes an important cytosolic C-terminal proline rich region (PRR). This PRR is characterized by a PxxP (Pro-Xaa-Xaa-Pro) motif and serves as a target for Src homology 3 (SH3) domains, which is present in the NOX cytosolic subunit $p47^{phox}$ and is crucial for the functional assembly of NOX (Groemping and Rittinger, 2005).

1.7.2 Cytosolic Subunits

The cytosolic subunits of NOX2 are $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$; composed of 526, 390, and 339 amino acids respectively (Bedard and Krause, 2007; Lin et al. 2007). The activation of NOX2 is orchestrated through a series of protein-protein and protein-lipid interactions leading to membrane translocation and interaction between the cytosolic subunits and $p22^{phox}$ of the cyt b_{558} complex. This translocation is initiated by conformational changes of $p47^{phox}$ induced by phosphorylation. This is followed by direct interactions with the SH3 domain of $p22^{phox}$ and accompanied by the union of the remaining cytosolic factors in a synergistic and cooperative conduct (Groemping and Rittinger, 2005). Organization of the cytosolic subunits involves several crucial electrostatic interactions between $p40^{phox}$ and $p67^{phox}$ PB1 domains to form a tight p40-$p67^{phox}$ complex that then binds the accessible SH3 domain of $p67^{phox}$ with the $p47^{phox}$ C-terminal PxxP motif in a reaction that takes place with a binding affinity of 20 nM (Groemping and Rittinger, 2005). Recent research suggests that remodeling of the actin cytoskeleton supports the translocation of cytosolic NOX subunits to the membrane.

Pharmacological studies showed that NOX activity is reduced both by actin polymerization and also by actin depolymerization, suggesting that actin filament dynamics is more important in NOX assembly than a net accumulation or loss of actin filaments (Roepstorff et al. 2008). This association is also implied due to the direct binding interaction between specific cytosolic NOX subunits two actin regulatory proteins: coronin (Grogan et al. 1997) and moesin (Wientjes et al. 2001). Furthermore, the C terminal region of $p47^{phox}$ is essential for both alpha- and beta-actin binding (Tamura et al. 2000).

1.7.3 Rac Protein

The small GTPase Rac isoforms 1-3 belong to the Rho-family of small GTPases that act as molecular switches in a multitude of signaling pathways. Their activation is directed by guanine nucleotide exchange factors (GEFs) that facilitate the exchange of GDP to GTP, which transforms the Rac proteins from their inactive GDP-bound state, to their active GTP-bound state. The two GTPase Rac proteins most important in the role of NOX activation are Rac1 and 2 (Bedard and Krause, 2007). While both Rac proteins are present in activated neutrophils, Rac1 expression is omnipresent and Rac2 expression is confined to hematopoietic cells (Bedard and Krause, 2007). The role of GTPase Rac in NOX2 activation is to interact with and activate $p67^{phox}$ succeeding the independent association of both proteins with cyt b_{558}. Although the precise mechanism of this interaction is unknown, research suggests the $p67^{phox}$ binding site on Rac is present as a highly conserved region on the C-terminal. Results obtained for $p67^{phox}$-Rac interaction

after Ala^{27} and Gly^{30} mutations were made on the Rac C-terminal suggested this region to be necessary for the protein binding and activation of $p67^{phox}$ (Kwong et al. 1993; Lapouge et al. 2000). Activation of $p67^{phox}$ is directly related to the initiation of enzymatic NOX activity, a crucial step to the completed complex formation and enzymatic functionality of NOX2.

1.7.4 Superoxide Production

NOX is selective for Nicotinamide Adenine Dinucleotide Phosphate (NADPH) over Nicotinamide Adenine Dinucleotide (NADH) as a substrate (Bedard and Krause, 2007). Once NOX is fully assembled, an electron transfer from the cytosolic side of the membrane to the outer side of the membrane is initiated. In the initial stages of the electron transfer, the electron acceptor on the cytosolic side of the membrane associates with NADPH and an electron is transferred from NADPH to flavin adenine dinucleotide (FAD), an energetically favorable step. This transfer occurs simultaneous to FAD binding amino acids $_{337}$HPFTLSA and $_{355}$IRIVGD (Vignais, 2002). In a second electron transfer, an electron leaves reduced $FADH_2$ for the iron center of the inner heme with the inner and outer asymmetrical hemes located in the transmembrane domains 3 and 5 bind histidines H101, H209, H115, and H222 (Finegold et al. 1996). Although this electron transfer from the inner to the outer heme is against the natural electromotive force, oxygen bound to the outer heme serves as an electron acceptor and enables the energetically favorable transfer of an electron from the inner heme to the outer heme. It is

this transfer that consequently leads to NOX generated superoxide (Bedard and Krause, 2007).

1.8 NADPH Oxidase and Lipid Raft Association

Eukaryotic cell membranes are composed of several active domains that are characterized by their distinct physical and biological properties. Together these domains are involved in many extracellular signal recognitions and serve as a platform for cellular signal transduction mechanisms. Of particular interest are cholesterol-dependent domains, also known as detergent resistant membranes (DRM) or lipid raft (LR) domains. LR domains are cholesterol and sphingolipid rich regions of the plasma membrane (PM) that are insoluble and thus stable in nonionic detergent lysates of mammalian cells (Brown and London, 2000). Elevated levels of cholesterol and sphingolipids provide essential structural framework, while other membrane proteins influence the ordering of the lipids to initiate raft formation (Tong et al. 2008). Cholesterol serves as a spacer between hydrocarbon chains of LR sphingolipids and functions as a dynamic glue that maintains LR assembly (Simons and Toomre, 2000). Due to the rigidity of cholesterols sterol group, it positions between the raft and non-raft phase of the membrane as it preferably interacts with LR sphingolipids; these lipids interact to form a liquid-ordered (Lo) phase in the bilayer (Chichili and Rodgers, 2009). LR domains serve as platforms for a multitude of enzymatic activity due to their cellular location, lipid components, and interaction with other membrane and cytosolic cellular domains.

Membrane associated proteins assist in cell-to-cell interactions, maintenance of cellular functions, and secretion and uptake of a variety of substances. They generally serve as therapeutic targets for a plethora of medical conditions including neurological disorders. Of particular interest are enzymes associated with LR membrane domains such as NOX. The NOX flavocytochrome b_{558} is suggested to interact with LR domains for complete functional assembly and successful superoxide formation (Vilhardt and Van Deurs, 2004). LR association with the actin cytoskeleton becomes of particular interest when studying NOX due to the translocation of cytosolic subunits to the flavocytochrome b_{558}. LR domains demonstrate scaffolding properties that are intimately linked to the recruitment and assembly of cytosolic NOX proteins with their membrane bound counterparts (Vilhardt and Van Deurs, 2004). It has been demonstrated that production of superoxide is stimulated via the formation of LR redox signaling platforms, implying that aggregated NOX subunits in LR platforms are functioning as active enzyme complexes when in the absence of inhibitors (Si Jin et al. 2008). NOX insolubility in nonionic detergent is likely a consequence of inclusion into LR domain, as the flavocytochrome b_{558}, regardless of cytosolic NOX subunits, associates with low-density DRM (Vilhardt and Van Deurs, 2004).

Membrane bound NOX subunits have been located in the LR compartment of neutrophil membranes, suggesting that the contribution of LR platforms are important to specific redox signaling events that orchestrate activation of the NOX enzyme system in neutrophils. The stimulation of NOX with IL-8, which acts to prime the oxidative burst, has been show to induce NOX components into LR domains and that these domains are

synergistically essential for the signaling events triggered by IL-8 (Guichard et al. 2005). NOX represents one of the first macromolecular enzymatic complexes to depend on LR domains for dynamic assembly and function (Klopfenstein et al. 2002). Enzymes, such as NOX, that associate with signaling pathways leading to disease, serve as targets for analyzing ROS induced oxidative stress. Understanding the relationship of NOX and LR domains of the plasma membrane is crucial to determine approaches for decreasing or inhibiting NOX activation and subsequent superoxide formation.

1.9 Stimuli and Activators of NADPH Oxidase

All Stimuli that induce assembly of NOX2 subunits induce lipid changes and kinase activities that support the functional assembly of NOX and ultimately superoxide production. The functional assembly of NOX and the subsequent formation of superoxide is essential for phagocytosis, a process of the human immune system used to remove pathogens, cellular debris, bacteria, fungi, parasites, and small mineral particles from cells. During exocytosis, internal phagosomes engulf foreign particles and ultimately deliver them to the lysosome for degradation (Stuart and Ezekowitz, 2005). Normal levels of superoxide produced by NOX activation plays a beneficial role in the human biological system however; over-stimulation or prolonged activation of NOX is likely to yield increased inflammatory effects. NOX can be activated, not only by an innate immune response, but also by external stimuli that can induce specific signaling cascades related to the regulation of NOX assembly. Understanding process of NOX activation by

various stimuli (Figure 3) may lead to a better understanding of how potential inhibitors can prevent or decrease NOX function.

1.9.1 Induced Phosphorylation

The release of arachidonic acid (AA) from membrane phospholipids during neutrophil activation has been associated with the activation of superoxide generation by NOX (Kapus et al. 1994). Cytosolic phospholipase A_2 ($cPLA_2$) plays a major physiological role in the release of intracellular AA from cellular membrane phospholipids. In Chinese-Hamster ovary (CHO) cells, the release of AA was shown to activate a proton flux through the N-terminal of $gp91^{phox}$ (Mankelow et al. 2003). The protein kinase c (PKC) activator, Phorbol 12-myristate 13-acetate (PMA) causes $cPLA_2$-mediated release of AA from its fatty acid precursor and subsequently induces NOX activity (Kramer et al. 1991). Although AA is the substrate for eicosanoid biosynthesis by cyclooxygenase, lipoxygenase, and cytochrome P450, it is established that AA itself acts upstream in the signaling pathway as the primary activator for NOX superoxide generation. It has been shown that superoxide generation is consistent, in the presence of AA, as well as when AA metabolite inhibitors are present (Kapus et al. 1994).

Cellular treatment with the phosphatatse inhibitor calyculin A, increases phosphorylation and membrane translocation of the $p47^{phox}$ NOX cytosolic subunit however; recent research shows this translocation is not accompanied by Rac translocation (Nigorikawa et al. 2004; Oommen et al. 2004). It is suggested that Rac recruitment to the oxidase system is a two-step process that triggers the translocation of

GDP-bound Rac in a PI3K-independent manner. The conversion of membrane-anchored Rac to its GTP-bound form depends on PI3K activity while the translocation of $p47^{phox}$ is dependent on other phosphorylation sources (Nigorikawa et al. 2004).

Lipopolysaccharide (LPS) is a bacterial cell wall component that stimulates NOX activation by inducting phosphorylatation of p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC) and phospholipase C (Rane et al. 1997). The coupling of post trimeric G-proteins to the formyl-methinyl-leucyl-phenylalanine (fMLP) receptor is known to stimulate superoxide production via catalysis of NOX (Stuart and Ezekowitz, 2005).

1.9.2 Cytokines

Tumor Necrosis Factor Alpha (TNFα) is a pro-inflammatory cytokine that plays critical roles in regulating the development and function of the immune system and also induces many cellular responses including necrotic and apoptotic signaling (Chen et al. 2007). TNFα binds to the TNFα-receptor-1 (TNF-R1) which is associated with an adaptor protein FAN and is implicated in ROS generation through NOX (Block et al. 2007).

The cytokine, Interleukin 1, beta (IL-1B), mediates an inflammatory response that results in ROS. IL-1B is known to increase cyclooxygenase-2 (COX2) in the CNS and may result in ROS through NOX activation. Specifically, IL-1B serves as a key stimuli in the self-perpetuating neurotoxicity cycle of reactive microgliosis (Block et al. 2007).

During this cycle IL-1B serves as a neurotoxic factor of direct neurotoxic insult, ultimately causing neuronal damage or death (Block et al. 2007).

1.9.3 Anesthetic

Ketamine is a dissociative anesthetic that when abused can lead to a syndrome indistinguishable from schizophrenia. Mice exposed to ketamine exhibit an increase in brain superoxide production derived from NOX activation (Behrens et al. 2007). Ketamine significantly increases the expression of $p22^{phox}$, and of NOX2, the NOX isoform found predominately in the CNS (Behrens et al. 2007). The increase of NOX-mediated superoxide formation as a result of ketamine exposure links this anesthetic to an inflammatory response and more specifically to NOX-mediated inflammation of the CNS.

1.10 NADPH Oxidase Inhibition

NOX inhibitors are important in elucidating enzyme mechanisms relevant to various diseases and to the development of new therapeutics for the prevention and treatment of pathologies associated with NOX-associated inflammation. It is important to analyze whether potential inhibitors act directly or indirectly on NOX. Here we categorize NOX 'inhibitors' as either pharmacological inhibitors, including Diphenylene iodonium (DPI), 4-(2-Aminoethyl) benzenesulfonylfluoride (AEBSF), Neopterin, GGF, Gp91ds-tat, and LR disruptors, or as natural product inhibitors such as 4-hydroxy-3-methoxyacetophenone (Apocynin), sinomenine, prodigiosins, and other natural products

found in a variety of fruits and vegetables. Importantly, all known NOX inhibitors at this time lack specificity or are extremely cytotoxic.

1.10.1 Pharmacological Inhibitors

1.10.1.1 Diphenyliodonium

Diphenyliodonium (DPI) is the most commonly used NOX inhibitor, known to inhibit all of the NOX isoforms as well as any other one-electron transporter reaction including but not limited to nitric oxide synthase, xanthine oxidase, mitochondrial complex 1, and cytochrome P-450 reductase. DPI abstracts an electron from an electron transporter to form a radical that inhibits the respective electron transporter through a covalent binding step. However, it is unclear if this iodonium radical formation occurs through interactions with the flavin group or the heme group (O'Donnell et al. 1993). Current research shows that osteoclasts expressing NOX1 and treated with DPI are inhibited from producing cytokines and support views that ROS produced by NOX is required for osteoclast differentiation (Lee et al. 2005).

1.10.1.2 AEBSF – 4-(2-Aminoethyl) benzenesulfonyl fluoride

AEBSF originally utilized as a serine protease inhibitor, was coincidently found to inhibit NOX by interfering with the association of the cytoplasmic subunit $p47^{phox}$. It is unclear whether AEBSF actually inhibits the $p47^{phox}$ subunit from translocating to the membrane bound subunits, or if it acts on signaling steps leading to the initial phosphorylation of $p47^{phox}$ (Diatchuk et al. 1997). Identifying the molecular mechanism

of this inhibitor would be beneficial in understanding the role of NOX in inflammation however AEBSF is highly unspecific for NOX inhibition and may encompass cellular effects that are unrelated to NOX and due to serine protease inhibition.

1.10.1.3 Neopterin

Neopterin is a pteridine generated by macrophages as a catabolic product of GTP. Neopterin inhibits the phagocyte NOX with an IC_{50} in the low micromolar range (Kojima et al. 1993). This inhibitor is not specific; it also inhibits xanthine oxidase, which makes its actual mechanism of NOX inhibition questionable.

1.10.1.4 GGF

Gly-gly-phen (GGF) is a dynorphin tri-peptide found to be the minimal peptide sequence required for neuroprotection against LPS-induced neurodegeneration in mixed neuron-glia cultures (Qin et al. 2005). Qin et al. (2005) have recently identified NOX as a possible critical high-affinity target for the femtomolar regulation of microglial activation (Qin et al. 2005). They found that neuroprotection and reduction of proinflammatory gene expression conferred by GGF is dependent on the presence of functional NOX. It is hypothesized that GGF inhibits NOX activity by binding to the gp91 subunit however; further research is needed to prove this. GGF is suggested to be neuroprotective through the inhibition of NOX thus this peptide may serve as a novel neuroprotective approach for inhibition of NOX-mediated free radicals.

1.10.1.5 Gp91 ds-tat – Blocking Peptide

The $p47^{phox}$-blocking peptide sequence, gp91ds-tat, is a chimeric peptide that was designed specifically to inhibit NOX2 by mimicking a sequence of NOX2 thought to interact with $p47^{phox}$. As a low-efficacy inhibitor, gp91ds-tat only inhibited neutrophil ROS generation by approximately 25 percent when used at 50 μM; the most potent superoxide inhibition occurred in the vascular system and was shown to inhibit superoxide generation through NOX1 and NOX4 inhibition (Bedard and Krause, 2007). This inhibitor is derived from a tat peptide of the HIV virus and affects NOX2 $gp91^{phox}$ by preventing interactions and binding of $p47^{phox}$. Gp91ds-tat is likely to act specifically on the oxidase, making it a unique tool for studying the involvement of NOX in *in vivo* models (Brandes, 2003).

1.10.1.6 Lipid Raft Disruptors

Disruption of LR domains may play a role in decreasing NOX function by disrupting the association of NOX cytosolic factors to their membrane-bound counterparts. However, LR disruption could also be detrimental to a cells ability to defend itself from pathogens. Research shows that superoxide production following cholesterol depletion is significantly decreased in intact cells (Vilhardt and Van Deurs, 2004). Due to the importance of cholesterol in LR integrity, a depletion of cholesterol causes redistribution of NOX and other raft proteins from a detergent-insoluble membrane compartment to a more soluble fraction (Foster et al. 2003). Perturbation of LR structural integrity using cholesterol-sequestering compounds such as Methyl-β

Cyclodextrin (MCD) increase delocalization of NOX subunits from LR domains and simultaneously decrease ROS production (Yang and Rizzo, 2007). A recent study by Vilhardt and Van Deurs (2004) demonstrates that association of flavocytochrome b_{558} with low-density LR domains is entirely dependent on cholesterol and that NOX activity on the cell surface is compartmentalized in a cholesterol-dependent manner (Vilhardt and Van Deurs, 2004). In addition, both membrane translocation of cytosolic proteins, and superoxide production was impaired in cholesterol-depleted cells. In conclusion, removal of LR cholesterol leads to dissociation of many proteins, including the NOX cytochrome b_{558}, from LR domains and renders them nonfunctional (27).

Other LR disruptors used in cardiovascular practice today that are potent, although nonspecific, are HMG-COa reductase inhibitors or statins. Statins attenuate NOX activation by depletion of isoprenoids, which prevents integration of Rac in the plasma membrane due to a loss of membrane anchoring (Wagner et al. 2000). Although these inhibitors are nonspecific, and their molecular mechanisms for inhibition are unclear, it is tempting to speculate that some of the benefits are consequential to the disruption of LR domains and may subsequently inhibit NOX activation (Wagner et al. 2000).

1.10.2 Natural Product Inhibition

1.10.2.1 Apocynin

Apocynin is a plant phenol discovered in the 1990's that acts as a low affinity inhibitor ($IC_{50} \sim 10$ μM) of the phagocyte respiratory burst (Simons et al. 1990). It has

been suggested that apocynin is metabolized by peroxidase to generate its inhibitory capacity and abolish the translocation of NOX cytoplasmic subunits (Stolk et al. 1994). Apocynin has been used in patients without signs of toxicity however, ill-defined characteristic of its inhibitory mechanism on NOX makes it undesirable for therapeutic development at this time. Unlike DPI, apocynin does not inhibit all forms of NOX. Specifically, Heumuller and co-workers proposed that apocynin is not an inhibitor of vascular NOX but that it acts as an antioxidant to scavenge free radicals so that its use resembles that of a NOX inhibitor. In a recent study using HEK293 cells over-expressing NOX1, 2, or 4, apocynin failed to inhibit superoxide anion generation detected by lucigenin Chemiluminescence (Heumuller et al. 2008). Apocynin did interfere with the detection of ROS in assay systems selective for hydrogen peroxide or hydroxyl radicals. These results suggest that apocynin acts as an antioxidant rather than a direct NOX inhibitor and that apocynin should not be used as an NOX inhibitor in vascular systems (Heumuller et al. 2008).

1.10.2.2 Sinomenine

Sinomenine (SN) is an alkaloid pure compound extracted from the Chinese medicinal plant, *sinomenium acutum* and used to treat inflammatory disease for many centuries (Feng et al. 1965; Finegold et al. 1996). Based on its molecular structure, SN belongs to a family of morphinans that are molecularly similar to morphine. Studies including several other morphinan compounds show neuroprotective anti-inflammatory characteristics. SN has been used in clinical trials and demonstrated efficacy for patients

suffering from rheumatoid arthritis. The pharmacological profile of SN includes immunosuppressant (Vieregge et al. 1999), arthritis amelioration (Liu et al. 1996), anti-inflammation (Liu et al. 1994), and protection against hepatitis induced by LPS (Kondo et al. 1994). Mediated through microglia, SN shows significant neuroprotection against both LPS and MPP^+ induced dopaminergic neuron neurotoxicity. SN acts to inhibit activated microglial NOX and in turn results in inhibition of a wide array of pro-inflammatory mediators (Wang et al. 2007). Although little is known about the molecular mechanism in which SN exhibits inhibitory effects, it may be a potential and safe therapeutic agent for the treatment of inflammatory-mediated neurodegenerative conditions (Qian et al. 2007).

1.10.2.3 Prodigiosin

Prodigiosin is a red pigment associated with the gram-negative bacteria, *Serratia Marcescens*. This red temperature regulated pigment plays a role in acidifying cellular vesicles by transporting H^+Cl^- across cellular membranes. Specifically, prodigiosins transport Cl^- anions across phospholipid membranes via H^+ influx or OH^- efflux. In a recent study a prodigiosin analogue was found to decrease PMA simulated $O2^-$, without antioxidant capability compared to SOD, and also decreased NOX induce $O2^-$ while having no effect on PKC activity (Nakashima et al. 2008). This study suggests that prodigiosins serve to inhibit NOX function through prevention of either Rac-GDP conversion to Rac-GTP or through prevention of $p47^{phox}$ phosphorylation. While the

mechanism remains unclear, it is safe to assume that prodigiosin plays a role in the inhibition of NOX via Rac or $p47^{phox}$ intervention.

1.10.2.4 Catechin

Increased NOX activity, measured by the expression of $p47^{phox}$, in the hippocampal CA1 brain region of rodents has been linked to intermittent hypoxia (IH) (Burckhardt et al. 2008) however, oral supplements of green tea catechin polyphenols (GTPs), have been shown to reduce neural susceptibility to IH during sleep in rodents. These findings inspire interest in potential therapeutic values of GTPs in conditions related to oxidative stresses. However, while the specific mechanism of GTPs is thought to effect $p47^{phox}$, its protective effects remain unresolved.

As the mechanisms of potential NOX inhibitors remain unknown, the challenge of studying the NOX enzyme persists. Many of the NOX inhibitors used today act indirectly or upstream of NOX rather than directly on the enzyme itself. Some of the inhibitors are unspecific and even cytotoxic. Discovery of specific NOX inhibitors as well as their mechanisms are crucial for future pharmacological intervention of NOX-mediated neuroinflammation.

1.11 Nutritional Intervention

Diets rich in fruits and vegetables have long been touted for their health benefits, often attributed to their high polyphenol content. The potential molecular targets of polyphenolic compounds provide a wide array of nutritional and pharmacological

implications and could help preventing the onset and progression of age-related diseases. Research from Joseph et al. (2003, 2009) suggests that dietary supplementation with fruits and vegetables high in polyphenols (e.g. blueberries, strawberries, and walnuts) can decrease the vulnerability to oxidative stress that occurs in aging (Joseph et al. 2009). Recently Krikorian et al. have shown blueberry supplementation to improve memory in the older adult population (Krikorian et al. 2010). A greater intake of high-antioxidant foods such as ginseng, walnuts, deep-colored vegetables and fruits may increase "health span" and enhance cognitive and motor function in the aging of humans (Joseph et al. 2009).

In addition to the high antioxidant capacity of flavonoids, a complex family of polyphenols found in many fruits and vegetables, neuroprotective qualities from these foods may also be attributed from non-antioxidant disruption of cellular processes as well (Ramassamy, 2006). Another mechanism to which phytochemicals could inhibit biochemical processes such as NOX-induced ROS is through lipid raft (LR) disruption. Fujimura et al. (2004) found a receptor associated with LR binding Epigallocatechin-3-O-gallate (EGCG), a potent flavonoid in green tea. This discovery introduces an exciting possibility that other flavonoids could influence cellular mechanisms by targeting lipid rafts. Tarahovsky and coworkers suggest that in addition to interaction with integral membrane proteins, hydroxyl or carboxyl moieties of flavonoids can interact with amino groups of sphingomyelin (Tarahovsky et al. 2008). Competitive incorporation of flavonoid compounds with cholesterol could also influence membrane fluidity and affect LR formation (Tarahovsky et al. 2008).

A study on the inhibitory effects of ginsenosides from the root of panax ginseng found ginsenosides to have no antioxidant effects when assayed for DPPH radicals or for superoxide anions. Five specific ginsenosides analyzed for NOX inhibition show inhibition of cytosolic subunit translocations to be dependent on stimuli used as well as the specific ginsenoside used as an inhibitor (He et al. 2008). This analytical method presents a beneficial strategy in determining the exact effects of various inhibitors and their possible roles in free radical regulation of NOX. It is important to identify NOX inhibitors but their mechanistic roles are crucial as well when attempting to combat NOX related disease.

1.12 Wild Alaska Bog Blueberry Extract Inhibits NOX

Blueberries are of great interest for nutritional interventions due to their high anthocyanin content and their potent antioxidant and anti-inflammatory effects (Krikorian et al. 2010). Blueberries were shown to alleviate cognitive decline in animal models and decrease ischemia-induced brain damage (Joseph et al. 2003; Sweeney et al. 2002). Our research demonstrates that crude extracts of wild Alaska bog blueberries inhibit NOX-mediated ROS. We have determined that the inhibition mechanism of wild Alaska bog blueberries is not through radical scavenging capability and that compounds in these berries specifically prevent the functional assembly of NOX by inhibiting the translocation of $p67^{phox}$ to the plasma membrane of neuroblastoma cells (figure 5).

1.13 Conclusion

The underlying mechanistic characteristics, functionality of NOX, and its implications in a vast array of diseases is poorly understood due to the lack of specific NOX inhibitors. The structural analysis of NOX remains incomplete and also contributes to the challenges of studying this multi-subunit enzyme. The potential for NOX inhibitors is endless as far as discovery, mechanism, and therapeutic application. The interest in determining NOX inhibitors has increased as implications for NOX-mediated ROS in a variety of pathologies becomes better defined. The few known NOX inhibitors used today have unknown functions and unspecific regulation. There are currently no potent and specific NOX inhibitors and the studies cited in this text, whose conclusions are based on current NOX inhibitors should be taken with some caution (Bedard and Krause, 2007).

Understanding the redox signaling linked to NOX is crucial to determine mechanisms in which NOX activation may be inhibited. It is now widely accepted that LR domains play a role, as signaling platforms, in the organization of components in membrane-directed intracellular signals (Klopfenstein et al. 2002). Because of this, LR disruptors cause decreased NOX function by inhibiting the association of NOX membrane bound subunits with LR domains and in turn prevent the translocation of PKC and NOX cytosolic factors to their membrane bound counterparts. Furthermore, nutrition intervention has been proven to help decrease inflammation of the CNS and reduce NOX related inflammation.

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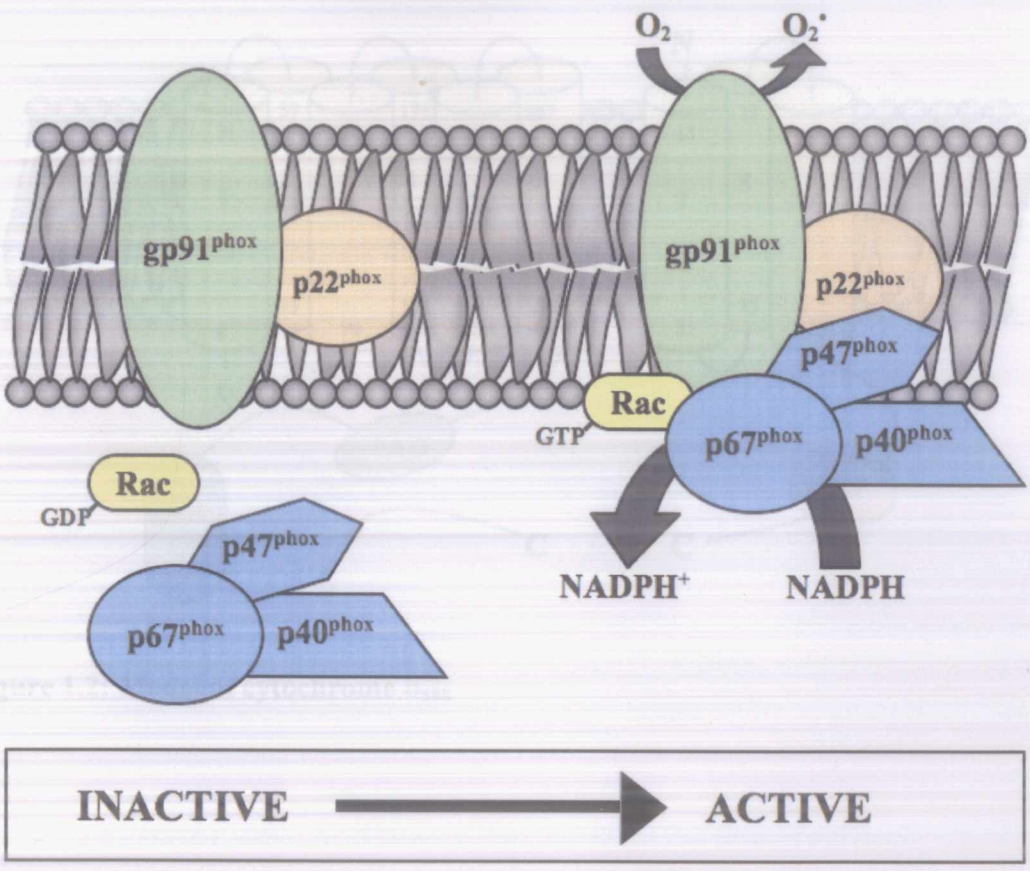

Figure 1.1: The functional assembly of NOX

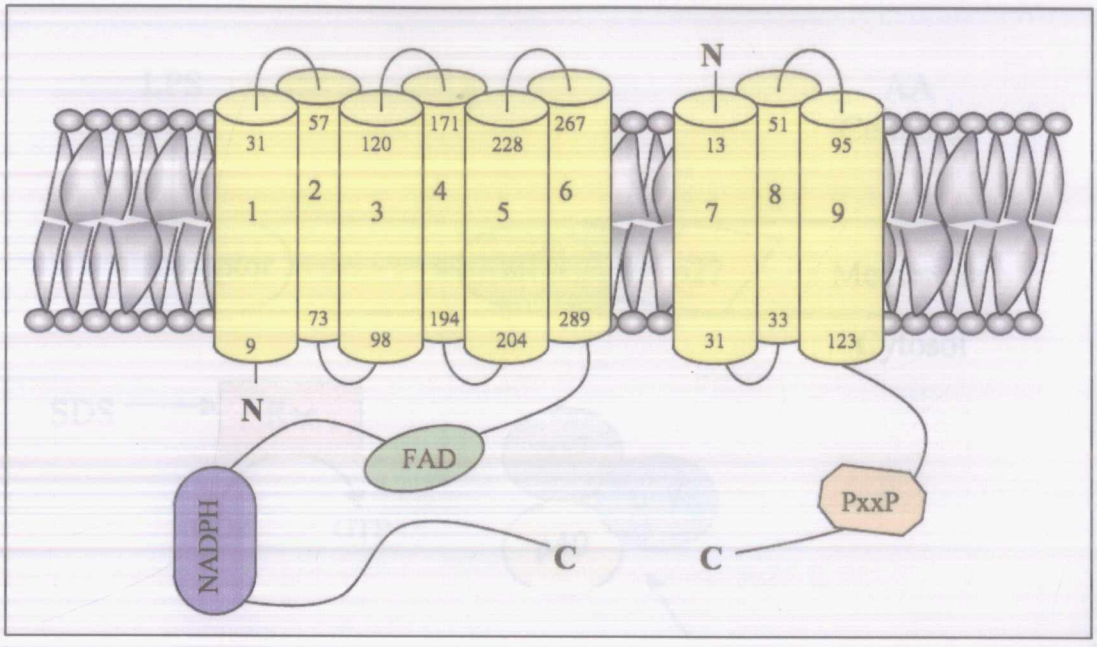

Figure 1.2: Model of cytochrome b_{588}

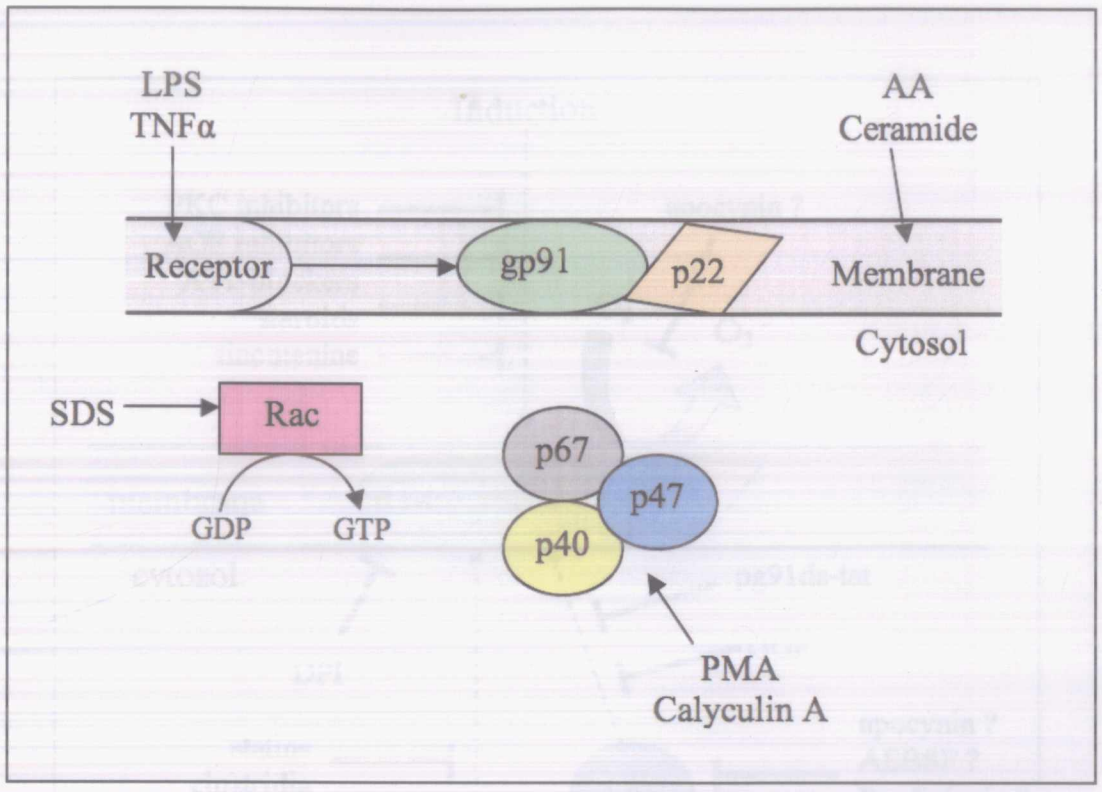

Figure 1.3: Cellular location of stimuli that influence the functional assembly of NOX

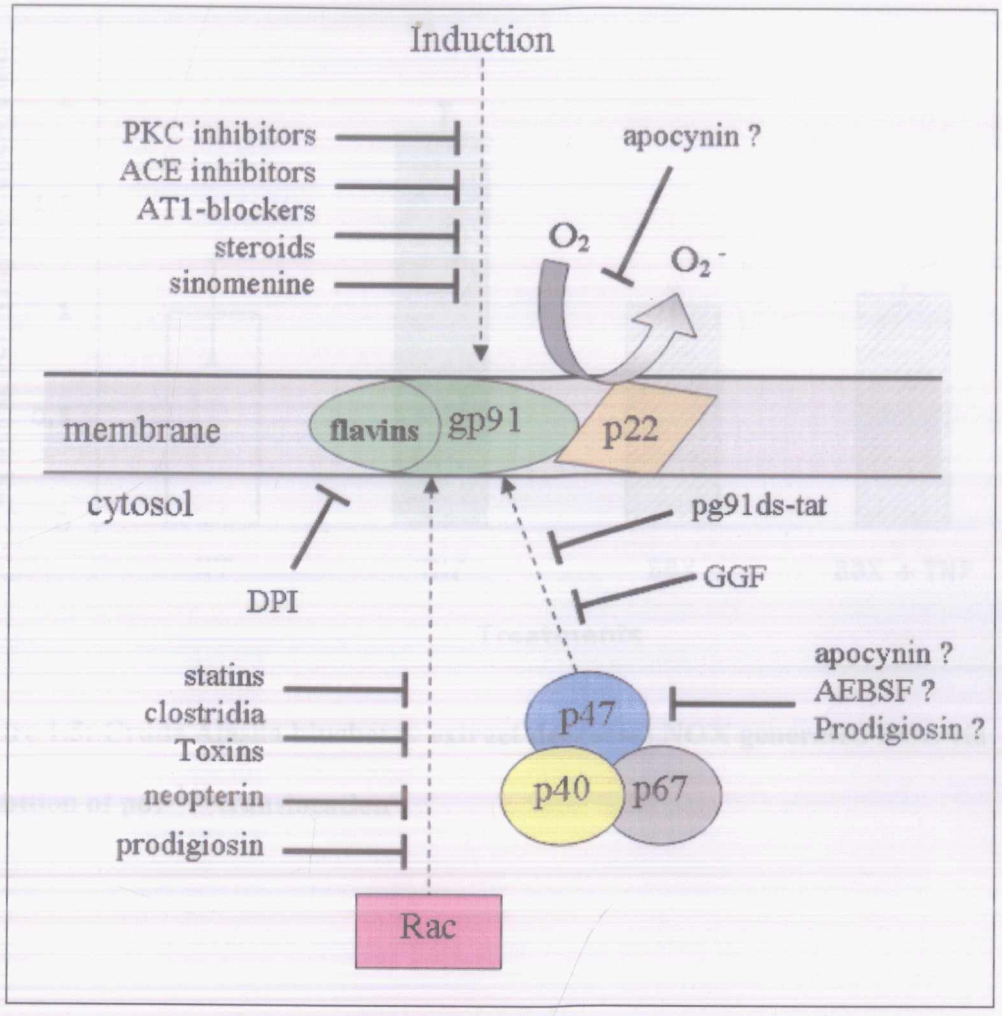

Figure 1.4: Potential NOX inhibitors

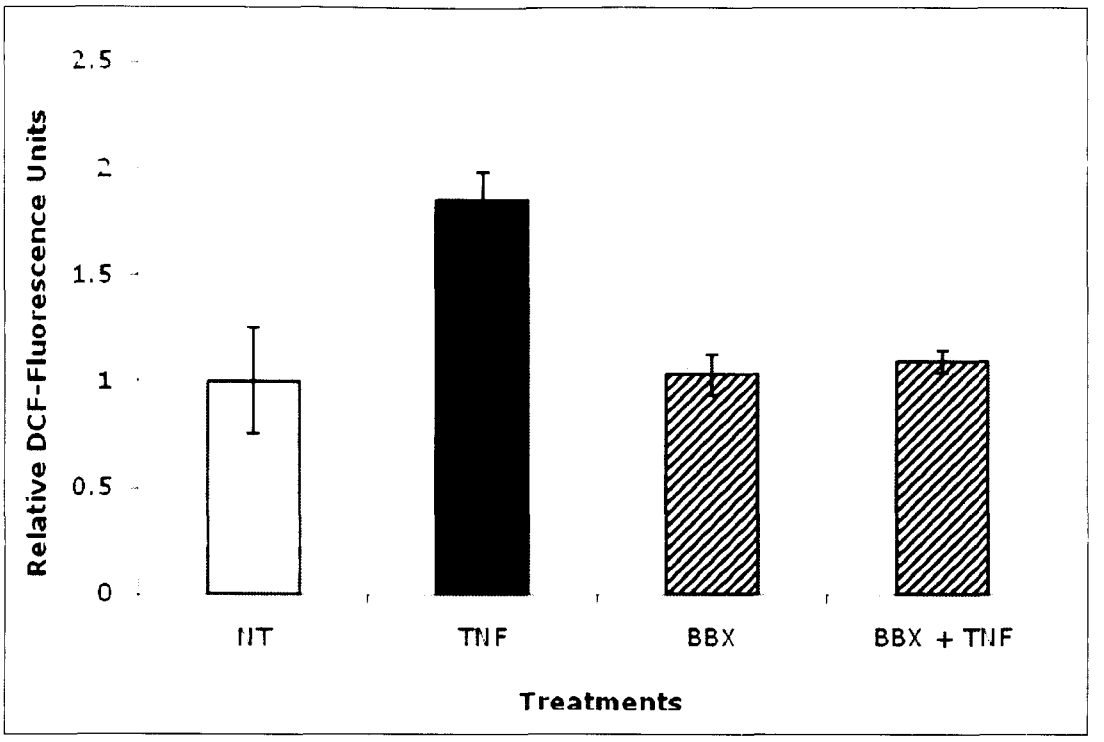

Figure 1.5: Crude Alaska blueberry extract decreases NOX generated ROS via inhibition of $p67^{phox}$ translocation

Chapter 2

Design of Thesis Research

2.1 Research Rationale

The role of NADPH Oxidase (NOX) in inflammation is vital and contributes to a vast array of pathologies including hypertension, chronic granulomatous disease, Amyotrophic Lateral Sclerosis, Neimann-pick disease, Alzheimer's, Parkinson's, and other forms of dementia as well as normal aging (Harraz et al. 2008). The functional assembly of this membrane bound, hetero-multimeric enzyme results in the formation of one particular reactive oxygen species (ROS) namely superoxide. The accumulation of superoxide has been interrelated to proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) that lead to the progression of neuronal degeneration. TNFα stimulates the activation of neutral Mg^{2+}-dependent sphingomyelinase (nSMase) in neuronal cells, which subsequently generates ceramide through hydrolysis of sphingomyelin, an abundant sphingolipid in neuronal plasma membranes. Ceramide-dependent cellular stress responses include excessive ROS formation, functional assembly of NOX, and increased oxidative stress.

Due to the lack of specific NOX inhibitors the molecular mechanism implicated in the pathway leading to neuroinflammation remains poorly understood. Identification of potential NOX inhibitors is critical to develop treatments that decrease or prevent NOX-mediated inflammation linked to disease. It is well documented that diets high in fruits and vegetables yield nutritional antioxidants known to decrease the effects of oxidative

stress and inflammation in disease and aging (Engelhart et al. 2002; Galli et al. 2002; Joseph et al. 2005). The beneficial properties of berries include bioactive phytochemicals, such as polyphenols, stilbenoids, and triterpenoids (Seeram, 2008). These nutritional components were found to have anti-aging effects and to decrease levels of proinflammatory cytokines associated with neuroinflammation (Krikorian et al. 2010; Paul et al. 2010). Recent research demonstrates inhibition of specific cellular targets by natural compounds in addition to the familiar antioxidant properties. In these studies, we investigate the effects of wild Alaska bog blueberries on NOX-mediated inflammation. For experimentation, wild blueberries from locations across the state of Alaska were fractionated and analyzed for their ability to inhibit ROS and other inflammatory biomarkers in neurons exposed to various stimuli.

2.2 Research Objectives and Aims

The impact of NOX on neuroinflammation renders it a prime target for both pharmaceutical and nutritional intervention. The objective of this thesis is to assess and hone beneficial natural compounds present in wild Alaska bog blueberries. The experimental design of this research is to examine the efficacy of specific fractions obtained from wild Alaska bog blueberries in a cellular model of neuroinflammation; SH-SY5Y human neuroblastoma cells exposed to TNFα. Ultimately, the attempt of this research is to use a nutrition-guided approach to identify compounds useful as specific NOX modulators that could serve as lead compounds for drug development.

2.2.1 Hypothesis One – Chapter 3

Crude blueberry extracts possess the ability to decrease magnesium-dependent neutral sphingomyelinase (nSMase) activity in neurons exposed to the proinflammatory cytokine, TNFα.

Aim one – To test whether crude blueberry extracts have the capacity to inhibit magnesium-dependent neutral sphingomyelinase activity.

Aim two – To test whether potential inhibitory actions of blueberry extracts are antioxidant in nature.

Aim three – To test the cytotoxicity of blueberry extracts on neuroblastoma cells.

2.2.2 Hypothesis two – Chapter 4

Blueberry fractions have the ability to inhibit NOX-mediated neuroinflammation in neurons exposed to TNFα and PMA irrelevant of their antioxidant capacity.

Aim one – To examine the efficacy of blueberry fractions with increasing polarity on NOX-mediated ROS production.

Aim two – To determine the oxygen radical scavenging capacity of blueberry fractions that inhibit NOX activity.

Aim three – To examine the efficacy of blueberry fractions with increasing polarity on NOX assembly, specifically translocation of $p67^{phox}$ to the plasma membrane.

2.2.3 Hypothesis three – Chapter 5

Ursolic acid, isolated from non-polar blueberry fractions, prevents the functional assembly of NOX by interfering with lipid raft platforms in the plasma membrane of neurons exposed to TNFα.

Aim one – To quantify the cytotoxicity of Ursolic acid.

Aim two – To quantify $p67^{phox}$ membrane translocation as a function of ursolic acid isolated from blueberries compared to commercially available ursolic acid.

Aim three – To determine the effects of ursolic acid on lipid raft formation in neuronal plasma membranes in the presence of TNFα.

2.3 Research Significance

We demonstrated that wild Alaska bog blueberries harbor natural compounds that specifically alter biochemical pathways beyond a passive oxygen radical scavenging capacity. We identified specific inhibition of nSMase and NOX hence disrupting a detrimental feedback mechanism linking sphingolipid metabolism and oxidative stress. Activation of both nSMase and NOX comprise the earliest biochemical process in inflammatory signaling located in the plasma membrane of neurons. Importantly, natural compounds identified and isolated from wild Alaska bog blueberries modulate the formation of lipid rafts in neuronal plasma membranes, which abolishes both nSMase and NOX activation and thus blunts ROS formation and ceramide generation.

These results illuminate the capacity of compounds found in berry fruit to serve as new avenues for therapeutics to antagonize NOX-mediated inflammation. Specifically,

ursolic acid among other compounds isolated from wild Alaska bog blueberries may serve to deregulate NOX activity and intervene with neuronal degeneration, with significant implications for acute, chronic, and psychiatric CNS pathologies, as well as general aging.

2.4 References

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Chapter 3

Alaska Wild Blueberry Extracts Inhibit a Magnesium-Dependent Neutral Sphingomyelinase Activity in Neurons Exposed to TNFα*

3.1 Abstract

The proinflammatory cytokine tumor necrosis factors α (TNFα) is key to initiating and orchestrating inflammation, which substantially contributes to the progression of many chronic and acute CNS pathologies. TNFα can stimulate a magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase) resulting in the accumulation of ceramide, a lipid messenger implicated in oxidative stress and apoptosis. Dietary polyphenols were shown to alleviate CNS inflammation largely attributed to their antioxidant properties. We found that preincubation of human SH-SY5Y neuroblastoma cells with aqueous or organic extracts prepared from Alaska wild bog blueberries completely negated Mg^{2+}-nSMase activation upon TNFα exposure. This specific and potent inhibition of Mg^{2+}-nSMase activity was non-antioxidant in nature. This study demonstrated for the first time that Alaska wild bog blueberries harbor the capacity to interfere with a key step in the progression of inflammation, the activation of Mg^{2+}-nSMase, in neuronal cells further providing evidence for the therapeutic potential of blueberries.

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* Current Topics in Nutraceutical Research, Gustafson SJ, Barth BM, McGill CM, Clausen TP, Kuhn TB, October 2007, Volume 5, Issue 4, pages 183-188

3.2 Introduction

The pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) exhibits pleiotropic effects in the developing, adult as well as injured nervous system (Merill 1992; Vitkovic et al. 2000; Allan and Rothwell 2001; Viviani et al. 2004). TNFα affects neuronal survival, contributes to the formation, repair and maintenance of synaptic connectivity, modulates outgrowth of neuronal processes, stimulates gene expression, and regulates neuronal redox-state. TNFα plays a key role in the initiation and orchestration of inflammatory in many chronic neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis), acute CNS injuries (stroke, traumatic injuries), and even psychiatric disorders (autism, schizophrenia) (Fontaine et al. 2002; Vargas et al. 2005; Keane et al. 2006; Lucas et al. 2006). TNFα is persistently secrete at high levels from reactive microglia and astroglia cells both prevalent in most CNS pathologies as well as in the aging CNS (Block et al. 2007). Interaction of TNFα with the TNFα receptors type I and II activates numerous cellular signal transduction pathways leading to stress kinase activation, oxidative stress, caspase activation, ceramide production, and ultimately apoptosis of neuronal cells (Locksley et al. 2001). Sphingolipids are highly prevalent in neuronal plasma membranes and serve as precursors for various lipid second messengers (Futerman and Hannun, 2004). For instance, ceramide is highly cytotoxic for most neuronal cell types and implicated in oxidative stress and apoptosis. Ceramide can be either synthesized *de novo* from the

palmitic acid or rapidly accumulates upon hydrolysis of complex sphingolipids by a TNFα-sensitive magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase). So far, three mammalian Mg^{2+}-nSMase have been characterized where Mg^{2+}-nSMase *2* is specifically expressed in mammalian brain (Clarke et al. 2006). Mg^{2+}-nSMase *2* is implicated in apoptosis, and inflammatory processes yet also in cell growth and differentiation. Inhibiting Mg^{2+}-nSMase *2* activity with GW4869 or via siRNA attenuated TNFα-stimulated apoptosis and generation of reactive oxygen species. Diets rich in fruit and vegetables were long known for the benefits to our health (Joseph, 2002). For instance, dietary intake of blueberries was shown to alleviate cognitive decline in animal models and decreases ischemia-induced brain damage (Mattson et al. 2002; Sweeney et al. 2002; Joseph et al. 2003). The active ingredients are largely attributed to the family of polyphenols, which act *in vitro* as highly effective antioxidants and reduce the progression of CNS inflammation (Ramassamy, 2006). However, many polyphenols are barely detectable in the CNS after dietary consumption implying the presence of compounds with non-antioxidant properties serving as specific and potent inhibitors in adverse inflammatory signaling pathways. We investigated whether Alaska wild bog blueberries (*Vaccinium uliginosum*) have the capacity to interfere with TNFα-stimulated inflammatory signaling pathways by specifically inhibiting Mg^{2+}-nSMase activity in neuronal cells.

3.3 Materials and Methods

3.3.1 Reagents: Recombinant human TNFα and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell Growth assay kit were purchased from Millipore (Temecula, CA). DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, and an Amplex Red Sphingomyelinase assay kit were received from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). All other reagents were purchased from Sigma (St. Louis, MO).

3.3.2 Cell Culture: SH-SY5Y human neuroblastoma cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml Penicillin and 100 U/ml Streptomycin (humidified atmosphere, 5% CO_2, 37°C) in 100 mm dishes (Falcon). Cells were harvested after incubation with trypsin (0.5 mg/ml)/EDTA (0.2mg/ml), briefly triturated, and plated into 6 well or 96 well tissue culture plates (200,000 cells per well or 60,000 cells per well, respectively). Prior to treatments, SH-SY5Y cells in 6 well pates were grown for 72 h whereas SH-SY5Y cells in 96 well plates were grown for 48 h.

3.3.3 Blueberry Extract Preparation: Alaskan wild bog blueberries (*Vaccinium uliginosum*) were harvested in the interior of Alaska at three distinct geographical locations (Lake Minchumina, Fox, and Fairbanks, Alaska) and stored frozen at -20oC.

Blueberries were lyophilized, crushed to a powder, and then extracted without hydrolysis either using an organic solvent or water. To obtain an organic blueberry extract (OBBX), 3.0 g of powdered blueberries were dissolved in 20 ml 70% acetone/30% water for 15 minutes with frequent agitation whereas an aqueous blueberry extract (ABBX) was prepared using 18 mΩ water. Insoluble material was removed by filtration and extracts were rotovaporized at 40° C (removal of volatile organics) prior to freezing and lyophilizing. The resulting powder form of extracts was stored at -20° C. OBBX or ABBX were reconstituted either in 70% acetone/30% water or 18 mΩ water only followed by a 1:20 dilution with 18 mΩ water immediately before addition to SHSY5Y cells.

3.3.4 Sphingomyelinase Assay: Subconfluent cultures of SH-SY5Y cells were pretreated with 5 μg/ml OBBX or ABBX for 2 h. After exchanging media, SH-SY5Y cells were exposed to TNFα (100 ng/ml) for 15 min, harvested in cold phosphate buffered saline and sonicated. Quantitative measurement of magnesium-dependent neutral sphingomyelinase activity was performed in cell lysates containing equal amounts of total protein (BCA assay) and using an enzyme coupled Amplex Red fluorescence assay kit (Invitrogen, Carsbad, CA). Briefly, nSmase activity in samples generates ceramide, which is further converted to phosphocholine. Choline is liberated by alkaline phosphatase and then converted to betaine and peroxide by choline oxidase. In last step,

horseradish peroxidase (HRP) reacts peroxide with amplex red to the highly fluorescent product resorufin measured at 590 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

3.3.5 Choline Oxidase Assay: Addition of choline to a mixture of choline oxidase, HRP, and Amplex red results in the formation of peroxide and subsequently the fluorescent product resorufin. In the absence of choline, the above mixture was preincubated with OBBX (5 μg/ml), ABBX (5 μg/ml and 75 μg/ml), 5 mM N-acetyl-L-cystein, or 2000 U/ml catalase for 15 min. The reaction was initiated by adding 50 μM choline. After 15 min incubation period (37^0 C), resorufin fluorescence was measured at 590 nm using a Multimode DTX 880 microplate reader.

3.3.6 Cytotoxicity Assay: Briefly, cultures of SH-SY5Y cells were serum deprived overnight and the supplement with blueberry extracts (5 and 75 μg/ml) for 48 h. Cytotoxicity was determined using an MTT cell viability assay according to manufacture's instructions (Chemicon). Formazan generation was measured using a Multimode DTX 880 microplate reader.

3.3.7 Statistical Analysis: Analysis of variance (ANOVA) was used to determine statistically significant differences between treatments ($p>0.05$). Post hoc comparisons of

specific treatments were performed using a Scheffe test to determine statistical significance based on the calculated ANOVA data. All error bars represent standard deviations of the mean (STD).

3.4 Results

In various cell types, TNFα stimulates a magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase) activity, which generates ceramide by hydrolyzing complex plasma membrane sphingolipids, a key step in the progression of inflammation. Dietary consumption of blueberries was shown to alleviate adverse effects of CNS inflammation. Thus, we tested whether organic or aqueous extracts obtained from Alaska wild bog blueberries (OBBX or ABBX, respectively) interfered with TNFα-stimulated activation of a Mg^{2+}-nSMase in SH-SY5Y human neuroblastoma cells. Mg^{2+}-nSMase activity was quantified utilizing an enzyme assay coupling ceramide generation in cell lysates to Amplex Red fluorescence. As show in Figure 1, SH-SY5Y cells revealed a dramatic increase in Mg^{2+}-nSMase activity (1.72+0.1, n=3, $p<0.05$) upon a 15 min exposure to 100 ng/ml TNFα compared to control cultures (1.00+0.29, n=3). In contrast, the presence of OBBX or ABBX (5 μg/ml each) negated TNFα-stimulated activation of Mg^{2+}-nSMase. The most potent inhibition of Mg_{2+}-nSMase activity was achieved with OBBX or ABBX from blueberries collected at Lake Minchumina (LM; 1.14+0.09, n+3, $p<0.05$ and 1.16+0.12, n=3, $p<0.05$, respectively). In comparison, inhibition of Mg^{2+}-nSMase was

less pronounced but still significant using OBBX and ABBX prepared from blueberries collected in Fox (FX; 1.3+0.2, n=3, $p<0.05$ and 1.37+0.22, n=3, $p<0.05$, respectively) or Fairbanks (FB; 1.12+0.18, n=3, $p<0.05$ and 1.31+0.03, n=3, $p<0.05$, respectively). Taken together our findings suggested that Alaska wild bog blueberries contain a potent inhibitor against Mg^{2+}-nSMase activity in human neuronal cells.

Since quantitative measurements of Mg^{2+}-nSMase activity in cell lysates relied on the formation of peroxide, inhibition of Mg^{2+}-nSMase in our experimental paradigm could have resulted from simple peroxide scavenging. Blueberries in general and Alaska blueberries in particular exhibit some of the highest antioxidant capacities measured (Prior 1998). We controlled for this alternative taking advantage of a direct choline oxidation assay. Addition of choline to a mixture of choline oxidase, HRP, and Amplex Red results in the formation of fluorescent resorufin. As shown in Figure 2, neither addition of ABBX nor OBBX to the direct choline oxidation assay interfered with peroxide production at a concentration (5 μg/ml) shown to completely inhibited Mg^{2+}-nSMase activity. However, increasing concentrations by 15 fold (75 μg/ml ABBX) revealed strong peroxide scavenging activity (FX; 66+4% +, n=8, $p<0.05$).
As expected, addition of 5 mM N-acetyl-L-cysteine (51+3%, n=8, $p<0.05$) or 2000 U/ml Catalase (51+2%, n=8, $p<0.05$) to the direct choline oxidation assay abolished peroxide generation compared to 3 μM peroxide, our positive control (42+4%, n=8, $p<0.05$). These findings suggest the non-antioxidant nature of Mg_{2+}-nSMase inhibition in our

blueberry extracts.

We further determined viability of human SH-SY5Y neuroblastoma cells upon prolonged exposure to blueberry extracts to verify the specificity of blueberry extracts in inhibiting Mg^{2+}-nSMase activity. Neither OBBX nor ABBX (5 μg/ml each) compromised SHSY5Y cell viability over a 48 h time period compared to control (Fig. 3). Moreover, SHSY5Y cell viability remained at control levels even when increasing concentrations of extracts 15 fold (75 μg/ml ABBX, FX). In contrast, UV irradiation of cells for 20 min drastically reducted cell viability as expected (positive control). This result demonstrates that blueberry extracts (5 μg/ml) did not compromise SH-SY5Y cell viability.

3.4.1 Extracts of Alaska wild bog blueberries inhibit Mg^{2+}-nSMase activity in neuronal cells

Blueberries were harvested at three different locations in the interior of Alaska: Lake Minchumina (LM), Fox (FX), and Fairbanks (FB). Both organic (70% acetone/30% water) and aqueous extracts were prepared (OBBX and ABBX, respectively) as described in materials and methods. Mg^{2+}-nSMase activity was quantitatively measured using an enzyme assay coupled to Amplex Red fluorescence. Exposure of human SHSY5Y neuroblastoma cells to 100 ng/ml TNFα for 15 min (T) significantly stimulated Mg^{2+}-nSMase activity (filled bar). In contrast, preincubation of SH-SY5Y cells with 5 μg/ml of ABBX (hatched bars) or OBBX (open bars) for 2 h prior

prior to TNFα stimulation abolished Mg^{2+}-nSMase activity and was indistinguishable from our control (Con; light gray bar). All values are normalized to control condition. Error bars represent standard deviations of the mean of three independent experiments (duplicates each) and statistical significance was determined at $p<0.05$ (ANOVA and pos hoc Scheffe test).

3.4.2 Inhibition of Mg^{2+}-nSMase activity results from non-antioxidant compounds in Alaska wild bog blueberry extracts

Peroxide generation was quantitatively measured via Amplex Red detection in a direct choline oxidation (ChOx). Compared to control (filled bar) neither OBBX nor ABBX exhibited peroxide scavenging capacity at a concentration of 5 μg/ml (open bars and hatched bars, respectively), which were shown to potently inhibit nSMase activity. Yet, addition of 75 μg/ml ABBX to the direct choline oxidation assay exhibited strong antioxidant capacity. As expected 5 mM N-acetyl-L-Cysteine (NAC) and 2000 U/ml catalase (CAT) significantly interfered with peroxide generation. Omitting choline in our assay resulted in virtually no fluorescence (n) and addition of peroxide (H) to Amplex red in the presence of HRP increased fluorescence (positive control). Error bars represent standard deviations of the mean from six independent experiments and statistical significance was determined at $p<0.05$ (ANOVA and post hoc Scheffe test). Extracts were prepared from Alaska wild bog blueberries harvested at Lake Minchumina (LM),

Fox (FX), and Fairbanks (FB).

3.4.3 Extracts of Alaska wild bog blueberries are not cytotoxic

Serum free cultures of SH-SY5Y cells were supplemented with OBBX and ABBX (5 μg/ml each) and maintained for 48 h prior to measuring cell viability (MTT assay). Neither OBBX nor AABX compromised cell viability at concentrations (5 μg/ml) shown to inhibit Mg^{2+}-nSMase activity compared to control conditions. Cell viability remained indistinguishable from control even when increasing ABBX to 75 μg/ml, a 15 fold excess (ABBX-75). However, UV exposure (20 min) of SH-SY5Y cells dramatically reduced cell viability (positive control, UV). Error bars represent standard deviations of the mean of at least four independent experiments and statistical significance was determined at $p<0.05$ (ANOVA and post hoc Scheffe test). We tested blueberries harvested from Lake Minchumina (LM), Fox (FX), and Fairbanks (FB).

3.5 Discussion

We demonstrated for the first time that Alaska wild bog blueberries have the capacity to inhibit a Mg^{2+}-nSMase activity in human neuronal cells upon exposure to the proinflammatory cytokine TNFα (Fig. 1). Moreover, our findings strongly suggested that Mg^{2+}-nSMase inhibition was non-antioxidant in nature (Fig. 2), extractable in either an organic solvent or water only, and benign to viability of neuronal cells over a

sustained time period. So far, three distinct Mg^{2+}-nSMase have been characterized in eukaryotes and, relevant to our study, Mg^{2+}-nSMase 2 is predominantly expressed in the mammalian CNS (Hofmann et al. 2000). Although not tested, our studies likely addressed Mg^{2+}-nSMase type 2 activity. First, experiments were performed in cholinergic and dopaminergic SH-SY5Y neuroblastoma cell line derived from a human CNS tumor. Second, this Mg^{2+}-nSMase was stimulated upon exposure of SH-SY5Y cells to TNFα (Fig. 1) as reported for the mammalian CNS Mg^{2+}-nSMase 2. Third, the pharmacological compound GW4869 inhibited TNFα-stimulated activation of Mg^{2+}-nSMase in SH-SY5Y cells (data not shown) in accordance with Luberto et al. (Luberto et al. 2002).

The capacity to inhibit Mg^{2+}-nSMase activity was present in both aqueous and organic extracts of Alaska wild bog blueberries harvested at three distinct locations (Lake Minchumina, Fox, Fairbanks). Although not statistically significant, aqueous or organic extracts prepared from Fox blueberries consistently contained higher potency in inhibiting Mg^{2+}-nSMase compared to either extracts prepared from Fairbanks blueberries of Lake Minchumina blueberries, respectively. These observed differences could arise from extracts not standardized according to a series of phytomarkers or actual distinct compositions of blueberries due to factors such as soil composition, water availability, or sun exposure for despite all three blueberry harvest locations lie within 200 miles of each other in the interior of Alaska. Clearly, future studies need to include a standardized

comparison among Alaska blueberries as well as blueberries from other US locations to determine whether significant differences in the potency of inhibiting Mg^{2+}-nSMase activity exist. The health benefits of a high dietary consumption of blueberries and many other fruits and vegetables are largely attributed to polyphenolic compounds with antioxidant properties. Since peroxide production was in integral step in our Mg^{2+}-nSMase activity assay, it was crucial to determine whether our blueberry extracts actively suppressed peroxide production. It is noteworthy that Alaska contain one of the highest antioxidant capacities measured (Wu 2004). Utilizing a direct choline oxidation assay, we detected no peroxide scavenging in either aqueous or organic blueberry extracts at concentrations of 5 μg/ml irrespective of the harvest location of blueberries. Importantly at a concentration of 5 μg/ml, Mg^{2+}-nSMase activity was completely blocked with either blueberry extract. Furthermore, aqueous or organic blueberry extracts inhibited Mg^{2+}-nSMase activity in a direct assay using a fluorescent Mg^{2+}-nSMase substrate (data not shown). Nevertheless, addition of aqueous and organic blueberry extracts at 15 fold higher concentrations significantly suppressed peroxide production indicative of their antioxidant properties. Conclusively, inhibition of Mg^{2+}-nSMase derived from a non-antioxidant compound present in Alaska wild bog blueberries.

Finally, inhibition of Mg^{2+}-nSMase activity could also be account for by an inherent cytotoxic effect of our blueberry extracts on SH-SY5Y cells. Some residual cell death is expected to occur under serum free conditions for 48 h even in controls.

Interestingly, both aqueous and organic blueberry extracts showed a strong tendency to increase cell viability compared to our control. Potentially, the high antioxidant properties of blueberry extracts were beneficial for cell viability analogous to B 29 supplementation of cultured hippocampal neurons. Activation of Mg^{2+}-nSMase and subsequent generation of ceramide is key to the progression of inflammation and increased of oxidative stress in the CNS common to many chronic neurodegenerative pathologies, acute CNS injuries, and even psychiatric disorders. Thus, Mg^{2+}-nSMase represents a pivotal target for therapeutic intervention. The fact that Alaska wild bog blueberries contain a potent Mg^{2+}-nSMase inhibitor could further substantiated the benefits of dietary consumption of blueberries to alleviate inflammation in the CNS (Wu et al. 2004; Noyan-Ashraf et al. 2005).

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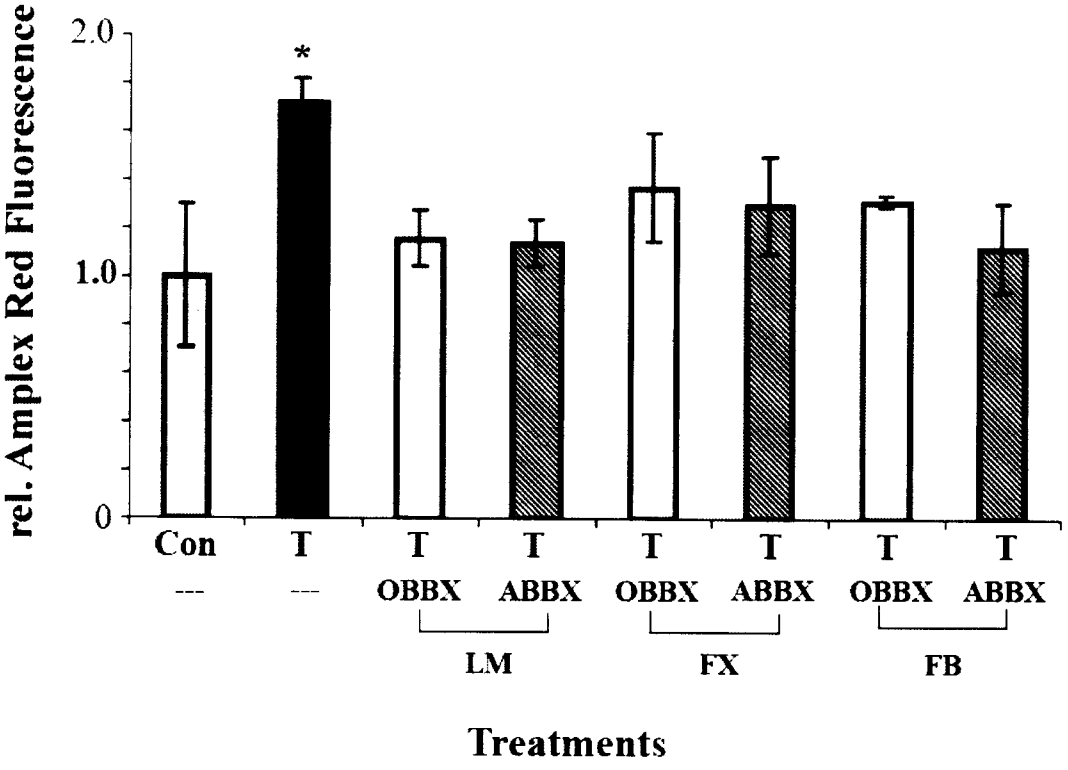

Figure 3.1: Extracts of wild Alaska bog blueberries inhibit Mg^{2+}-nSMase activity in neuronal cells.

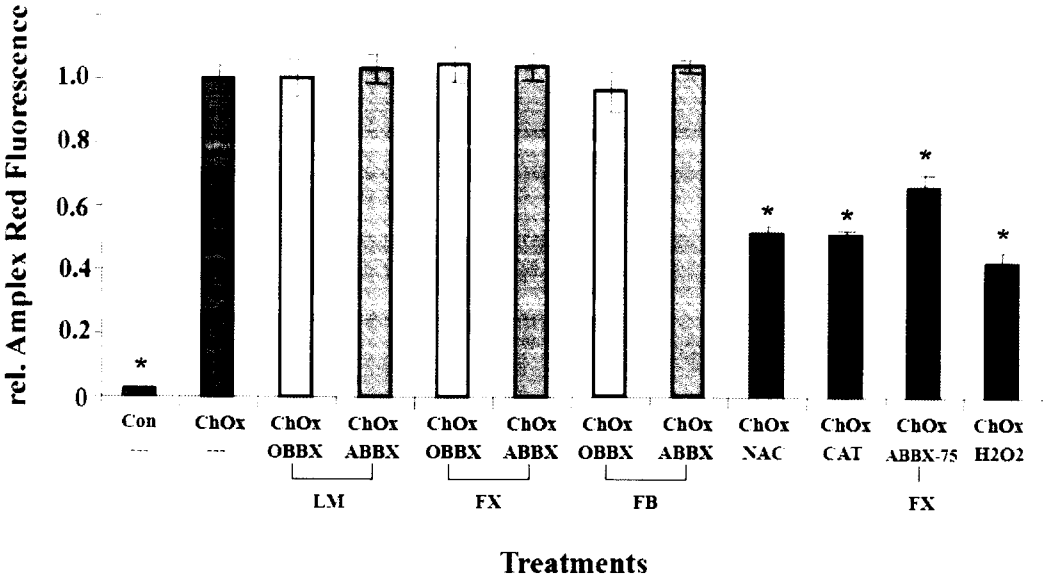

Figure 3.2: Inhibition of Mg^{2+}-nSMase activity results from non-antioxidant compounds in wild Alaska bog blueberry extracts.

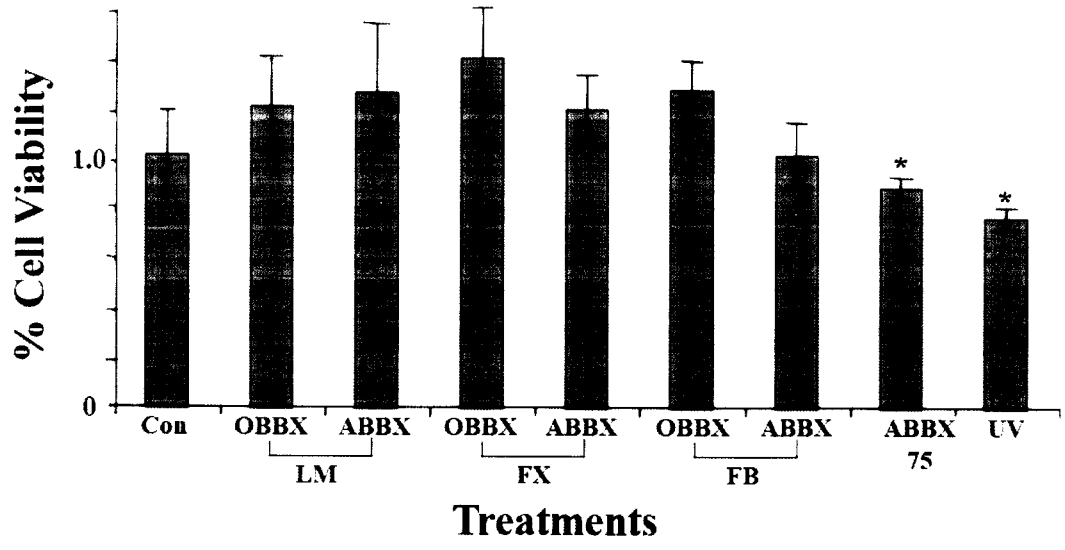

Figure 3.3: Extracts of wild Alaska bog blueberries are not cytotoxic.

Chapter 4

A Non-antioxidant Compound Present In A Non-polar Blueberry Fraction Inhibits NADPH Oxidase-Mediated Neuroinflammation*

4.1 Abstract

The functional assembly of NADPH Oxidase (NOX) results in the production of superoxide, one type of reactive oxygen species (ROS) (Bedard and Krause, 2007; Heyworth et al. 2003). Accumulation of ROS in the central nervous system (CNS) increases neuronal stress and oxidative damage that accompany NOX related neuroinflammatory processes (Lambeth et al. 2008). The coexistence of inflammation and oxidative stress is prevalent in acute, chronic, and psychiatric conditions and is largely responsible for neuronal degeneration. Molecular compounds found in fruits and vegetables are of interest as potential inhibitors for key enzymes of neuroinflammatory pathways. Here, we report that a non-antioxidant component present in non-polar fractions obtained from wild Alaska bog blueberries inhibits the translocation of the NOX cytosolic subunit, $p67^{phox}$, to the plasma membrane in neuroblastoma cells. In turn, the inhibition $p67^{phox}$ translocation prevents the functional assembly of NOX and blunts NOX-mediated ROS formation in neuroblastoma cells exposed to TNFα. We demonstrate that the mode in which this translocation is inhibited is by modulation of lipid raft (LR) signaling platforms in neuroblastoma cells. These findings provide

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evidence for the therapeutic potential of blueberries in inhibiting NOX assembly and ultimately in decreasing superoxide formation and the progression of CNS inflammation.

4.2 Introduction

The NADPH Oxidase (NOX) enzyme consists of two integral membrane proteins, $gp91^{phox}$ and $p22^{phox}$, otherwise known as the flavocytochrome b_{558} (cyt b_{558}) (Lin et al. 2007), and three cytosolic subunits, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$ (Bedard and Krause, 2007). NOX activation is orchestrated through a series of protein-protein interactions that lead to the translocation of the cytosolic subunits to the membrane where they interact with $p22^{phox}$ of the cyt b_{558}. This translocation is initiated by conformational changes of $p47^{phox}$ via phosphorylation. The functional assembly of NOX is pertinent for the innate immune response known as phagocytic burst, which serves as a host defense mechanism against microbial infection by producing superoxide (Stuart and Ezekowitz, 2005). While basal levels of NOX-mediated ROS are beneficial cellular systems, prolonged NOX activity possesses less profitable inflammatory effects. The CNS is extremely susceptible to the hazardous consequences of oxidative damage (Olanow, 1993) and inflammation associated with NOX-mediated ROS. NOX isoforms are expressed throughout mammalian tissue and play significant roles in the onset and progression of many diseases. NOX participation is evident in general aging, chronic granulomatous disease, hyperthyroidism, cardiovascular physiology, vascular ailments including hypertension, and a plethora of neurodegenerative diseases including Amyotrophic Lateral Sclerosis

(ALS), Neimann-Pick disease, Alzheimer's, Parkinson's, and other forms of dementia (Vigone et al. 2005; Harraz et al. 2008).

Inflammatory mediators are key to the progression of neurodegeneration in CNS pathologies. The proinflammatory cytokine tumor necrosis factor-α (TNFα) is released by activated Microglia and stimulates Mg^{2+}-dependent neutral sphingomyelinase (nSMase) activity. This activity rapidly generates ceramide via hydrolysis of sphingomyelin (Figure 4.1) and is prevalent in neuronal plasma membranes. The bioactive lipid messenger ceramide is implicated in oxidative stress, by means of NOX, as well as apoptosis in CNS neurons (Cutler et al. 2004; Ichi et al. 2009). Alternatively, phorbol 12-myristate-13-acetate (PMA) directly stimulates the assembly of NOX. PMA activates protein kinase C (PKC), which results in the phosphorylation of $p47^{phox}$ crucial to the functional assembly of NOX.

Foods containing nutritional antioxidants have long been considered to decrease the effects of oxidative stress and inflammation coupled to neurodegenerative diseases (Engelhart et al. 2002; Galli et al. 2002; Joseph et al. 2005). The beneficial properties of berries have been related to their bioactive phytochemicals, including polyphenols, sillbenoids, and triterpenoids (Seeram, 2008). The vast array of nutritional components that blueberries possess were shown to have anti-aging effects and to decrease levels of pro-inflammatory cytokines associated with neuroinflammation (Paul et al. 2010). Understanding the mechanism of NOX inhibitors may lead to a better understanding of the function of this membrane bound enzyme and provide insight in new therapeutics for decreasing or preventing inflammation of the CNS. Here, we investigated the effects of a

non-antioxidant component, present in non-polar blueberry fraction (NP_{BB}), on NOX activation in neuroblastoma cells exposed to TNFα.

4.3 Materials and Methods

4.3.1 Reagents

DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, TMB (3,3',5,5'-tetramethylbenzidine), H_2DCFDA (2',7'-dihydrodicholorfluorecein diacetate), Alexa fluor 555 Lipid Raft labeling kit and NuPAGE running and transfer buffers were purchased from Invitrogen (Carlsbad, CA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell Growth assay kit was obtained from Millipore (Temecula, CA). Anti- $p67^{phox}$ polyclonal antibody and goat anti-rabbit IgG secondary antibody conjugated to HRP were from Abcam (Cambridge, MA). BCA Protein assay kit and 1-step NBT/BCIP were from Pierce (Rockford, IL), PMA from Biomol (Plymouth, PA), and fetal bovine serum was received from Atlanta Biologicals (Atlanta, GA). Recombinant human TNFα was received from ProSpec (Rehovot, Israel). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

4.3.2 Cell Culture

SH-SY5Y human neuroblastoma cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, and 1% GlutaMax-1, (humidified atmosphere, 5% CO_2, 37°C) in 100 mm dishes

(Falcon). Cells were harvested with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml), briefly triturated, and plated into poly-D-lysine coated glass cover slips in 35 mm dishes (confocal microscopy) or into tissue culture treated 6 well or 96 well plates. Cells were grown for 24 hours to 40% (confocal microscopy) or 80% confluency and then serum starved overnight in DMEM, 1% GlutaMax-1, 100 U/ml penicillin and 100 U/ml streptomycin (humidified atmosphere, 5% CO_2, 37°C) prior to experimentation.

4.3.3 Blueberry Extract Preparation

Blueberries (*Vaccinium uliginosum*) were harvested and stored frozen (-20°C). Blueberries were lyophilized and then crushed to a powder. Approximately 20 grams of sample was fractionated over a silica column in 500 ml segments with a mobile phase of 80/20 dichloromethane/methanol. Extracts were filtered, roto-vaporized at 40°C to remove volatile organics, frozen and then lyophilized again. The powder extracts were stored at -20°C until reconstituted immediately prior to use in a 70/30-acetone/water solution, and diluted 1:20 into deionized (18 mega Ω) water as our stock solutions of non-polar and polar blueberry fractions.

4.3.4 Quantification of Reactive Oxygen Species Production

Cells were grown in 6 well tissue culture plates and serum deprived overnight. Cultures were incubated (1 h) either with DPI (10 μM), NAC (1 mM), AEBSF (1 mM), GW (13.8 μM), or 5 μg/ml of blueberry fractions were administered in conjunction with H_2DCFDA (1μM). Cultures were washed and exposed (1 h) to TNFα (200 ng/ml), PMA

(400 ng/ml), or no stimuli (control, nt) in serum free media – during which H_2DCFDA is oxidized to DCF by peroxide. Cell were washed with 1X PBS, lysed (2 M Tris-Cl pH 8.0, 2% SDS, 10 nM Na_3VO_4) and 200 ul of lysates were transferred to a black 96 well plate (falcon). ROS formation was quantified as maximum DCF-fluorescence intensity using a Beckman Coulter Multimode DTX 880 microplate reader (495 nm excitation filter, 525 emission filter). All values were corrected to the amount of protein and normalized to control (max relative DCF fluorescence/ ug protein).

4.3.5 Choline Oxidase Assay

Addition of choline to a mixture of choline oxidase, HRP, and amplex red results in the formation of peroxide and consequently the fluorescent product resorufin. To assay for interference of peroxide production, 5 μg/ml and 75 μg/ml of non-polar (NP_{BB}) and polar (PO_{BB}) fractions or catalase (2000 U/ml) was incubated with 1X reaction buffer (1 h). Prior to the addition of 50 μM choline (15 mins, 37° C) the above mixture was added to the preincubated treatments (15 mins). Resorufin fluorescence was measured at 590 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

4.3.6 Cytotoxicity Assay

Briefly, cultures of SH-SY5Y cells were serum deprived overnight followed by treatments of Milli-Q H_2O (positive control), or 5, 30, or 75 μg/ml blueberry extracts (non-polar and polar) for 48 hours. Potential toxic effects on cell viability were monitored using a MTT assay according to manufacture's instructions (Millipore). Formazan

generation was measured by an absorbance reading at 595 nm with a reference filter of 620 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

4.3.7 ELISA Assay for $p67^{phox}$

Cells were grown in 100 mm dishes to confluency and serum deprived overnight followed by 1 hour treatments with 5 μg/ml non-polar or polar blueberry fractions. Cells were then stimulated with TNFα (200 ng/ml) or PMA (400 ng/ml) for 1 hour, harvested in a sucrose buffer (20 mM Tris-HCL, 2 mM EDTA, 0.5 EGTA, 2 mM AEBSF, 25 μg/ml Leupeptin, 0.33 M Sucrose, pH 8.0), sonicated, and fractionated into cytosolic and plasma membrane fractions. Briefly, suspensions were centrifuged at (15 min, 16,000 x g) and the supernatant was removed (cytosolic fraction). The pellet was then resuspended and sonicated in a buffer containing 20 mM Tris-HCL, 2 mM EDTA, 0.5 mM EGTA, and 2 mM AEBSF, pH 8.0. Following centrifugation (15 min, 16, 000 x g), the supernatant was collected (plasma membrane). Protein content of membrane fractions was quantified using a BCA assay (Pierce) and 20 μg/ml of protein in 1X TBST was added to a high protein absorbent 96 well plate (Falcon) for 12 hours at 25°C. Wells were blocked with 5% w/v BSA in 1X TBST (1 h), and incubated with $p67^{phox}$ primary antibody (3 μg/ml, 4°C, overnight). Next, the cells were rinsed with 1X TBST and incubated with corresponding secondary antibody (1:2000, 45 mins, 25°C) prior to addition of tetramethylbenzidine (TMB) (100 ul/well, 10 mins). Colorimetric analysis was performed using a Beckman Coulter Multimode DTX 880 microplate reader by measuring absorbance at 620 nm.

4.3.8 Gel Electrophoresis and Western Blotting

For SDS-polyacrylamide gel electrophoresis, equal amounts of total protein (5 μg total membrane protein) were loaded on a 10% agarose gel and separated (125 V, 50 W, 40 mA) using NuPAGE MES running buffer (Invitrogen). Proteins were transferred to nitrocellulose membranes (100 V, 350 mA, 50 W, 1 hr) using NuPAGE transfer buffer (Invitrogen) and then blocked 1 hr with 5% w/v BSA in 1X TBST. Membranes were incubated overnight with primary antibody (1:500 in 1X TBST, anti-$p67^{phox}$, 4° C), washed (1X TBST), and then incubated with the corresponding secondary antibody (1:5000 in 1X TBST, 1 hr, room temp) conjugated to alkaline phosphatase. Immunoreactivity was detected by colorimetric detection using NBT/BCIP (Pierce).

4.3.9 Confocal Microscopy

Human SH-SY5Y neuroblastoma cells were grown on poly-lysine coated glass cover slips (0.13 mm thick German glass) in medium for 24 h. Cells were serum-starved overnight and incubated 1 h with non-polar or polar blueberry fractions (5 μg/ml) prior to acute addition of TNFα (200 ng/ml) or PMA (400 ng/ml). Cultures were labeled with Alexa fluor Lipid Raft Labeling kit 555 (Invitrogen) according to manufactures instructions. Next, cultures were fixed for 20 mins with 4% paraformaldehyde at 4°C, permeabalized for 20 mins with 0.3% TritonX-100 in 1X PBS at 4° C, and then blocked with 1% normal goat serum in 1X PBS. Cultures were incubated with primary antibody (anti-$p67^{phox}$, 1:200, overnight, 4°C) in 0.1% TX-PBS, washed (1X PBS), and incubated with secondary FITC-conjugated goat-anti-rabbit antibody (1:200, 2 h, RT, light

protected). After rinsing with 1X TBS, cover slips were mounted with PVA-DABCO (Peterson, 2001). After drying overnight at room temperature, slides were stored at 4°C until image analysis. Images were acquired (63x, oil, Plan Fluor) with a Zeiss confocal microscope LSM 510 equipped with a He/Ne laser and an Argon laser using filter combinations for Alexa 555 (554 excitation, 570 emission) and FITC (494 excitation, 517 emission). Zeiss LSM Software was used for image acquisition and analysis. For each treatment condition, random fields of view were analyzed and 3 cells from two independent sets of experiments ($n = 6$) were examined for the presence of lipid raft platforms (red), $p67^{phox}$ (green) membrane translocation, and colocalization (yellow).

4.3.10 Statistical Analysis

Data are presented as means±standard deviations except for data in Figure 5 (means±standard error of the mean). Significant differences between treatments and within multiple groups (*$p<0.05$) were examined using analysis of variance (ANOVA) followed by Tukey's Post hoc analyses performed using Statistical Analysis System (SAS) software.

4.4 Results

4.4.1 Non-polar blueberry fraction inhibits oxidative stress of neuroblastoma cells exposed to TNFα and PMA

CNS inflammation accompanied by oxidative stress is prevalent in normal aging and in many chronic neurodegenerative disorders, including Alzheimer disease (Apelt et

al. 2004; Armitage et al. 2009). Recent findings implicate members of the NOX family as pivotal sources of oxidative stress (Armitage et al. 2009). It was demonstrated that nutritional supplementation with blueberry reduces aging associated deficits, such as oxidative stress, in the CNS (Krikorian et al. 2010). The underlying mechanistic characteristics of NOX inhibitors are either unspecific or unknown, leaving NOX function and its implications in a vast array of diseases inadequately understood. The proinflammatory cytokine TNFα stimulates NOX via an upstream mechanism that generates ceramide as a key step in the progression of inflammation. In contrast, PMA elicits $p47^{phox}$ phosphorylation and thus a direct NOX activation. It is important when identifying a NOX inhibitor to look at both types of NOX activation to help conclude the inhibition mechanism.

We explored whether blueberry supplementation would harbor the capacity to inhibit NOX dependent ROS formation in neuronal cells exposed to TNFα or PMA. In this study SH-SY5Y human neuroblastoma cells, loaded with DCF, were incubated with pharmacological inhibitors or blueberry fractions from extracts, prior to exposure of TNFα or PMA.

Crude wild Alaska blueberry extract inhibited TNFα mediated ROS formation compared to control (inset, Figure 4.2A). Importantly, a non-polar fraction (NP_{BB}) obtained from Alaskan blueberry extract was sufficient to completely inhibit ROS formation in SH-SY5Y cells exposed to TNFα (1.03 ± 0.04, n=4) indistinguishable from control (0.99 ± 0.07, n=4) and in contrast to a polar blueberry fraction (PO_{BB}) obtained from Alaska blueberry extract (2.53 ± 0.1, n=4), which were ineffective (Figure 1A). In

the absence of blueberry fractions, TNFα strongly stimulated ROS formation (1.91 ± 0.06, n=4). Widely used, yet unspecific NOX inhibition (10μM DPI: 1.07 ± 0.06, n=4; 1mM AEBSF: 0.99 + 0.04, n=4), ROS scavenging (1mM NAC: 0.9 ± 0.06, n=4) and blocking nSMAse activity (13.8μM GW4869: 0.68 ± 0.26, n=4) all negated TNFα stimulated ROS formation. Since PMA is a well-documented, strong activator of NOX, we tested whether NP_{BB} would also inhibit PMA stimulated ROS formation in neuronal cells (Figure 4.2B). As expected exposure of SH-SY5Y cells to 400 ng/ml PMA resulted in ROS formation (1.93 ± 0.30, n=4), compared to control (1.00 ± 0.08, n=4). Both 10 μM DPI and 1 mM AEBSF (0.84 ± 0.16, n=4 and 0.52 ± 0.13, n=4) as well as 1 mM NAC (0.96 ± 0.22, n=4) all abolish PMA-stimulated ROS formation. Indeed, NP_{BB} also inhibited PMA stimulated ROS formation (1.07 ± 0.16, n=4) whereas PO_{BB} was ineffective (1.85 ± 0.25, n=4). Taken together, the potency of Alaska blueberries to inhibit NOX dependent ROS formation in neuronal cells, exposed to TNFα or PMA, partition exclusively into a non-polar blueberry fraction.

4.4.2 Non-polar blueberry fractions lack ROS scavenging capacity

Blueberries are known to have exceptionally high antioxidant capacity measured as ORAC levels (Wang et al. 2008). Since quantitative measurements of ROS rely on the oxidation of fluorescent indicator DCF by peroxide it was necessary to examine antioxidant capacities of NP_{BB} and PO_{BB}. We employed an enzyme-dependent ROS formation (choline oxidation) assay to determine the ROS scavenging properties of NP_{BB} and PO_{BB} (Gustafson et al. 2007). As shown in Figure 4.3, NP_{BB} fraction both at 5 μg/ml

or 75 μg/ml exhibited no ROS scavenging capacity indicated by the complete lack of interference with peroxide formation (2.92 ± 0.27, n=5 and 3.16 ± 0.25, n=5), compared to conditions without choline (C, 1.00 ± 0.07, n=5) or presence of catalase (0.98 ± 0.04, n=5). In the absence of blueberry fractions or catalase, addition of choline generated formation of fluorescent resorufin indicating peroxide production (4.08 ± 0.05, n=5). It is noteworthy that PO_{BB} fraction revealed ROS scavenging capacity at 75 μg/ml (1.9 ± 0.25, **$p<0.05$), compared to NP_{BB} (75 μg/ml), which was not observed at 5 μg/ml PO_{BB} fraction (3.08 ± 0.15, n=5). Notably, neither non-polar or polar blueberry fraction compromised neuronal viability at concentration from 5 μg/ml as high as 75 μg/ml (Figure 4.4). In summary, the potency of NP_{BB} to inhibit NOX-dependent ROS formation was not the result of an ROS scavenging capacity or reduced neuronal viability and hence suggested a potential interference with the function assembly of NOX.

4.4.3 Non-polar blueberry fractions interfere with functional NOX assembly in plasma membrane

NOX2 is comprised of two membrane subunits ($gp91^{phox}$ and $p22^{phox}$) and three cytosolic subunits $p67^{phox}$, $p40^{phox}$, and $p47^{phox}$ (Bedard and Krause, 2007). Generation of ROS by NOX requires functional assembly of cytosolic and membrane subunits. The degree of $p67^{phox}$ translocation from the cytosol to the plasma membrane is a widely accepted indication of functional NOX assembly. We investigated whether NP_{BB} fraction would interfere with NOX assembly using an ELISA assay directed against $p67^{phox}$. As shown in Figure 4.5A, exposure of SH-SY5Y cells to 200 ng/ml of TNFα for 30 minutes

caused a significant accumulation of $p67^{phox}$ in the plasma membrane (1.60 ± 0.28, n=4), compared to control (1.00 ± 0.07, n=4). Most importantly, NP_{BB} completely abolished $p67^{phox}$ accumulation in plasma membrane (0.92 ± 0.06, n=4) as opposed to PO_{BB} fractions (1.8 ± 0.06, n=4). Utilizing the same experimental design, we directly stimulated functional NOX assembly using PMA (2.10 ± 0.2, n=8), compared to control (1.00 ± 0.11, n=8) (Figure 4.5B). As determined with TNFα as a stimulus, NP_{BB} (5 μg/ml) also negated PMA mediated translocation of $p67^{phox}$ to plasma membrane (1.14 ± 0.14, n=8) in contrast to PO_{BB}, which was ineffective (2.00 ± 0.21, n=8). Next, SH-SY5Y cells were exposed to TNFα (200 ng/ml) or PMA (400 ng/ml) in the presence or absence of NP_{BB} and PO_{BB} and translocation of $p67^{phox}$ to the plasma membrane was analyzed by western blotting (Figure 4.6). As expected, 200 ng/ml TNFα or 400 ng/ml PMA stimulated translocation of $p67^{phox}$ into plasma membrane (4.77 ± 0.74 and 2.80 ± 0.50, n=3, respectively). The effects of blueberry fractions on translocation of $p67^{phox}$ to the plasma membrane was corroborated with NP_{BB} negating functional NOX assembly in the presence of TNFα and PMA (1.07 ± 0.23 and 1.07 ± 0.14, n=3, respectively). PO_{BB} fraction was ineffective at inhibiting $p67^{phox}$ translocation when induced by TNFα (3.3 ± 0.65, n=3) or PMA (3.4 ± 0.12, n=3).

4.4.4 Functional assembly of NOX is associated with lipid raft platforms

Lipid raft microdomains exist as organized structures for signaling pathways (Brown, 2002) and recent evidence shows that functional NOX assembly is dependent on the formation of these signaling platforms. We explored whether NP_{BB} and PO_{BB} were

capable of modulating the formations of lipid raft (LR) platforms using confocal microscopy (Figure 4.7). Here we demonstrate that SH-SY5Y cells exposed to NP_{BB} (Figure 4.7B) resulted in minimal raft formations as well as nominal $p67^{phox}$ translocation and colocalization when compared with control (Figure 4.7A). Exposure to PO_{BB} under the same conditions resulted in few LR formations, $p67^{phox}$ translocation to the membrane and colocalization of LR and $p67^{phox}$ compared to control (Figure 4.7A and C). As expected, SH-SY5Y cells stimulated with PMA (Figure 4.7D) resulted in translocation of $p67^{phox}$ to the cellular membrane and discontinuous LR and colocalization puncta. Similarly, cells treated with TNFα (Figure 4.7G) exhibited $p67^{phox}$ translocation, inclusive and continuous LR formations, and colocalization of $p67^{phox}$ and LR. PO_{BB} treated cells exposed to PMA (Figure 4.7F) showed significant $p67^{phox}$ translocation as well as LR and colocalization congruent with that of PMA treated cells (Figure 4.7D). As expected, TNFα induced continuous lipid raft formation, $p67^{phox}$ translocation to the plasma membrane, and colocalization of $p67^{phox}$ and lipid rafts (Figure 4.7G) compared control (Figure 4.7A). More importantly, we found that cells pre-treated with NP_{BB} prior to TNFα exposure (Figure 4.7H) exhibit modulated LR platforms revealed as discontinuous colocalization in addition to minimal $p67^{phox}$ membrane translocation. PO_{BB} was ineffective in modulating TNFα induced LR platforms and $p67^{phox}$ translocation (Figure 4.7I). These results confirm western blotting data (Figure 4.6) in addition to suggesting the inhibition of $p67^{phox}$ translocation by NP_{BB} coincides with a modulation of LR platforms.

4.5 Discussion

We demonstrated for the first time that NP_{BB} fractions of Wild Alaska bog blueberries contain the ability to inhibit NOX-mediated ROS in neuroblastoma cells exposed to direct and indirect stimuli PMA and TNFα, respectively (Figure 4.1). Additionally, our findings reveal NP_{BB} inhibition of NOX-mediated ROS is not linked to antioxidant properties. In fact, we show that NP_{BB} is non-antioxidant in nature (Figure 4.2) even at concentrations that are fifteen-fold (75 μg/ml) higher than our inhibitory concentration (5 μg/ml). Moreover, our findings suggest that the mode of inhibition by NP_{BB} is through the prevention of $p67^{phox}$ translocation from the cellular cytosol to the plasma membrane (Figure 4.5). We show that NP_{BB} interferes with this crucial translocation in the presence of both TNFα and PMA (Figure 4.5A and 4.5B) and thus inhibits the functional assembly of NOX. The ability of NP_{BB} to inhibit the function assembly of NOX correlates with a decrease in NOX-mediated ROS production in neuroblastoma cells exposed to TNFα or PMA (Figure 4.2A and 4.2B). To account for decreased NOX activity associated with possible cytotoxic effects of both blueberry fractions used in our experimentation, we measured the cell viability of SH-SY5Y cells treated with NP_{BB} and PO_{BB} (Figure 4.4). Neither fraction showed compromised cell viability effects at our standard concentration (5 μg/ml) or up to fifteen-fold this concentration (75 μg/ml). Western blot analysis (Figure 4.6) shows that both TNFα and PMA increase relative intensity of $p67^{phox}$ in plasma membrane fractions compared to untreated SH-SY5Y cells. Furthermore, we demonstrate that NP_{BB} decreases the relative intensity of $p67^{phox}$ in plasma membrane in the presence of both TNFα and PMA while

PO_{BB} was ineffective. It is known that the proinflammatory cytokine TNFα is a strong activator of NOX-mediated ROS formation in neuronal cells (Barth et al. 2009) and research shows that TNFα is also instrumental in the formation of lipid rafts (Doan et al. 2004). Using immunocytochemistry and confocal microscopy we assessed the effects of NP_{BB} and PO_{BB} on lipid raft formation (Figure 4.7). Here we verified the results of Figure 4.6 using cellular imaging and in addition showed that NP_{BB} modulates LR platforms. In conclusion, the mode of NOX inhibition demonstrated by NP_{BB} may be due to the modulation of LR platforms and in turn the prevention of $p67^{phox}$ to the plasma membrane.

Our results contribute to the usefulness of a dietary regimen high in fruits and vegetables; a concept that has long been known to yield health benefits. In particular, the high levels of polyphenolic compounds found in blueberries attribute to decreased ROS and CNS inflammation, alleviation of cognitive decline in animal models, reduced ischemia-induced brain damage (Joseph et al. 2003; Sweeney et al. 2002), and even improved memory in humans (Krikorian et al. 2010). In addition, wild Alaska bog blueberry extracts were shown to inhibit neutral sphingomyelinase activity in neurons exposed to TNFα with no detectable antioxidant capacity (Gustafson et al. 2007). This phenomenon, along with our results, exploits the idea of nutritional intervention and natural product oriented drug development. Our results demonstrate that components other than antioxidants, in blueberries, may also yield health benefits when incorporated into a diet.

The NOX enzyme is linked to a vast array of diseases and neuronal aging that are all associated with inflammation. More specifically, NOX is attributed to increased oxidative stress in the CNS common to many chronic neurodegenerative pathologies (Bedard and Krause, 2007). Determination of specific NOX inhibitors is critical for the development of potential therapeutics to combat the detrimental effects linked to NOX-mediated ROS. While NOX inhibitors remain scarce, this study gives hope to nutritional intervention and disease prevention through diet. The paradigm presented here provides evidence of a non-antioxidant compound present in non-polar blueberry extract that inhibits NOX-mediated ROS and validates the efficacy of dietary constituents, other than antioxidants.

4.6 Acknowledgements

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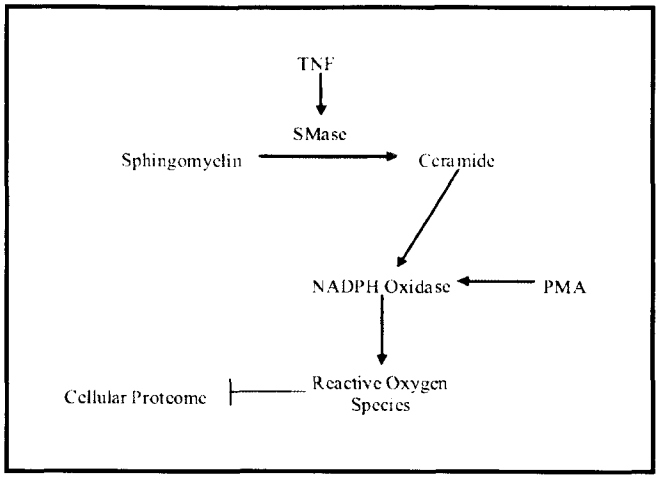

Figure 4.1: Activation of NADPH oxidase. TNFα indirectly stimulates NADPH Oxidase by liberating ceramide from sphingomyelin in a signaling cascade that increases lipid raft platform formation and interactions with the cellular cytoskeleton to allow the translocation of the NADPH Oxidase cytosolic subunits to the membrane subunits enabling functional assembly. Another mode of NADPH Oxidase activation occurs in the presence of PMA, which increases phosphorylation of the NADPH Oxidase cytosolic subunit, $p47^{phox}$ and subsequent activation of the oxidase in a more direct fashion.

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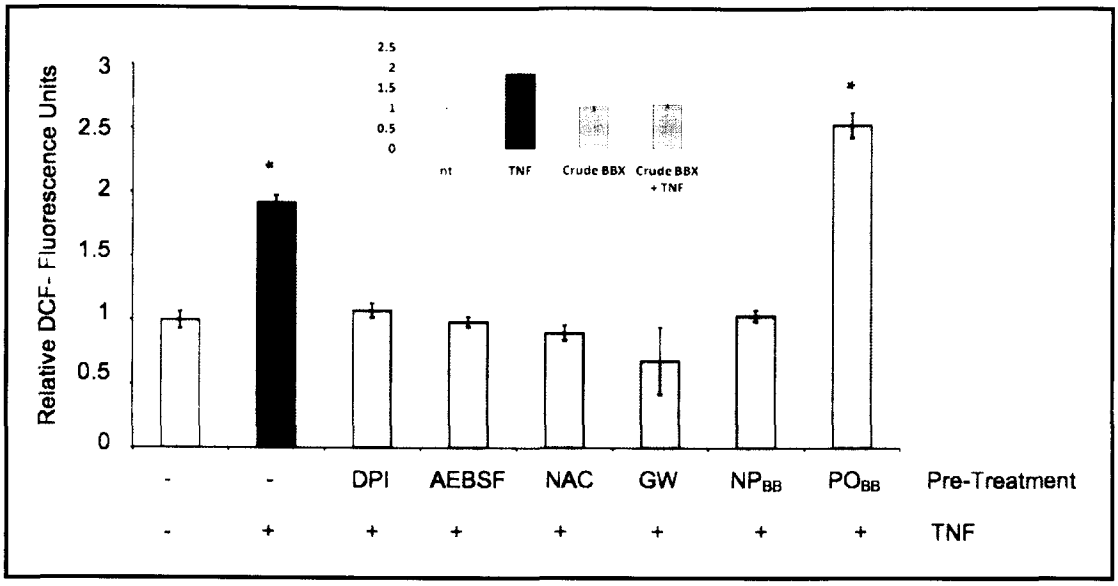

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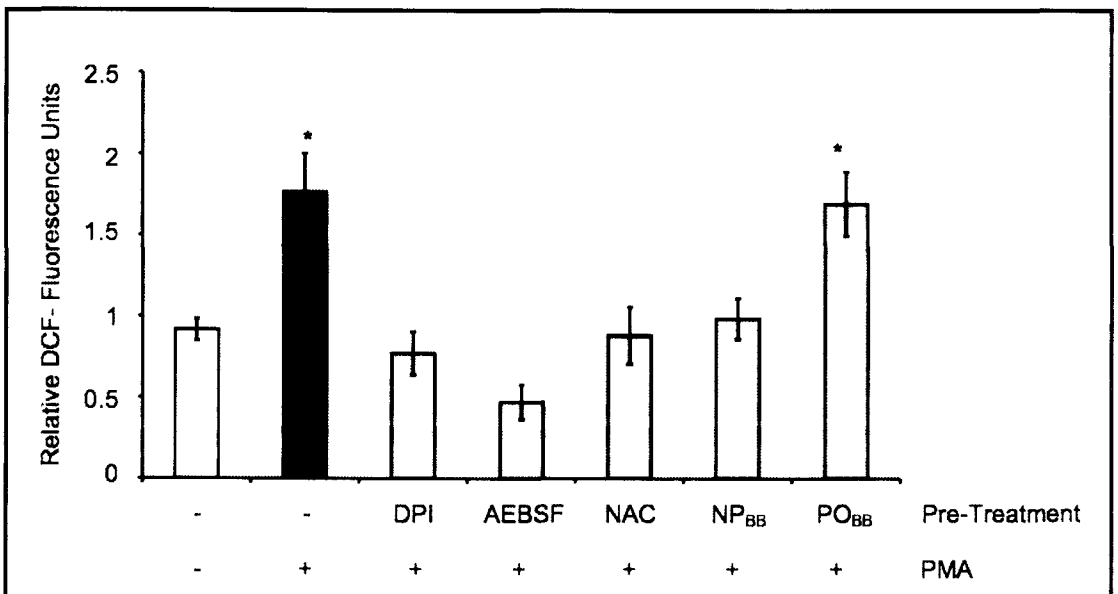

Figure 4.2: Non-polar blueberry fraction inhibits oxidative stress in neuroblastoma cells exposed to TNFα and PMA. SH-SY5Y human neuroblastoma cells were loaded for 1 h

with 50 μM 2',7'-dihydrodicholorfluorecein diacetate (H_2DCFDA) in the presence or absence of pharmacological inhibitors, non-polar (NP_{BB}), or polar (PO_{BB}) blueberry fractions. Next, cultures were exposed for 1 h to 200 ng/ml TNFα or 400 ng/ml PMA and ROS formation was quantified as increases in maximum DCF-fluorescence intensity in whole cell lysates. All measurements were normalized to the average maximum fluorescence intensity under control conditions. (A) TNFα exposure of SH-SY5Y cells resulted in a significant increase in ROS formation (filled bar), which was negated by a presence of 10 μM DPI, 1 mM AEBSF, 1 mM NAC, 13.8 μM GW4869 (gray bars), or NP_{BB} compared to control (open bar) where as PO_{BB} was ineffective in inhibiting ROS formation. (B) PMA exposure of SH-SY5Y cells also resulted in a significant increase in relative maximum DCF-fluorescence (filled bar), which was abolished in the presence of NOX inhibitors (10 μM DPI, 1 mM AEBSF), antioxidants (5 mM NAC) (gray bars), or NP_{BB} compared to control (open bar). PMA-evoked ROS formation was not effected in the presence of PO_{BB}. All data represent the mean of at least four independent experiments ± standard deviations (SD), and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

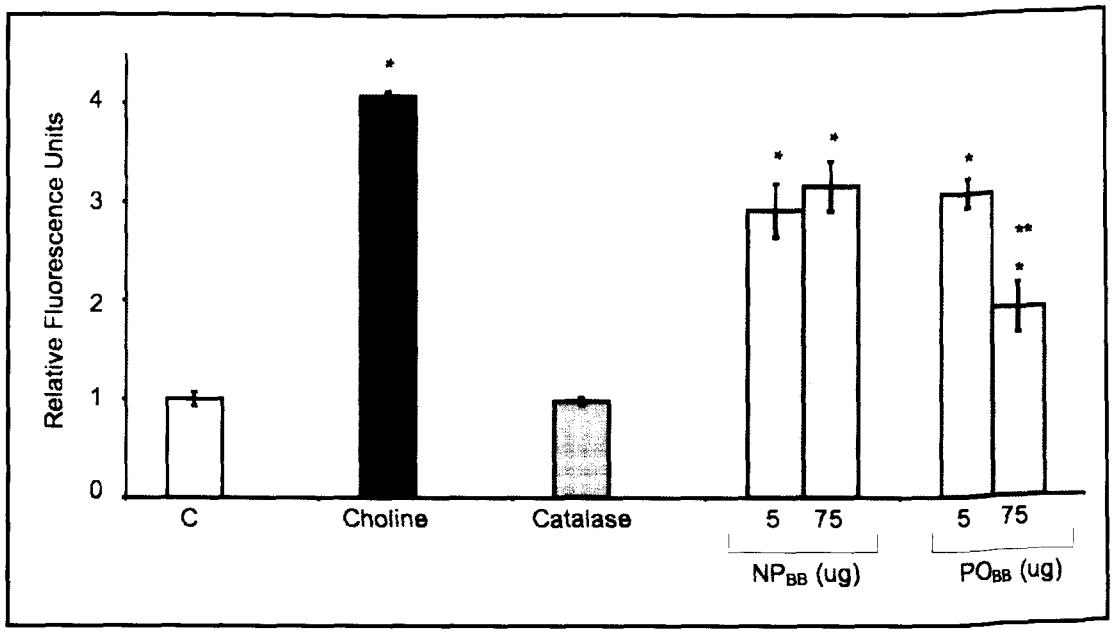

Figure 4.3: Non-polar blueberry fractions lack ROS scavenging capacity. Peroxide generation was quantified via Amplex Red in a direct choline oxidation assay through the conversion of choline to betaine and peroxide via choline oxidase. All fluorescence measurements were normalized to omission of choline, our control (C, open bar). Addition of choline (filled bar) resulted in peroxide formation which was completely abolished in the presence of 2000 U/ml catalase (gray bar). Inclusion of 5 μg/ml or 75 μg/ml non-polar blueberry fraction (NP_{BB}) in the choline oxidation assay were both ineffective in scavenging peroxide formation, yet 5 μg/ml was sufficient to completely abolish ROS formation in SH-SY5Y cells exposed to TNFα or PMA. In contrast, 75 μg/ml of polar blueberry fraction (PO_{BB}, $^{**}p<0.05$ significantly different than 75 μg/ml NP_{BB}) exhibited significant peroxide scavenging capacity, which was not observed at a fifteen-fold lower concentration (5 μg/ml). All data represent the mean of at least four

independent experiments ± standard deviations, and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

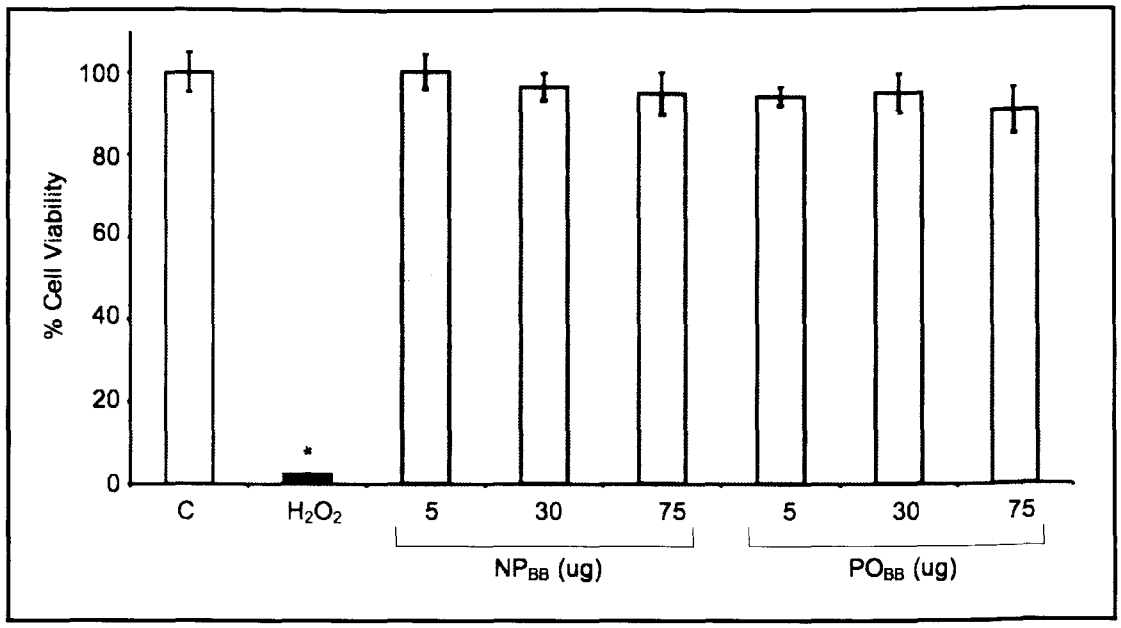

Figure 4.4: Non-polar and polar blueberry fractions are not cytotoxic. Serum free cultures of SH-SY5Y cells were supplemented with 5, 30, and 75 μg/ml of non-polar blueberry fraction (NP_{BB}) or polar blueberry fraction (PO_{BB}) for 48 h. Cell viability was measured using a MTT assay and all values were normalized to control (C, open bar). Neither NP_{BB} or PO_{BB} blueberry fractions compromised cell viability at concentrations effective in negating ROS formation in SH-SY5Y cells exposed to TNFα (200 ng/ml) or PMA (400 ng/ml) compared to our positive control (H_2O, 48 h, filled bar). Data represent the mean of eight independent experiments ± standard deviations, and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

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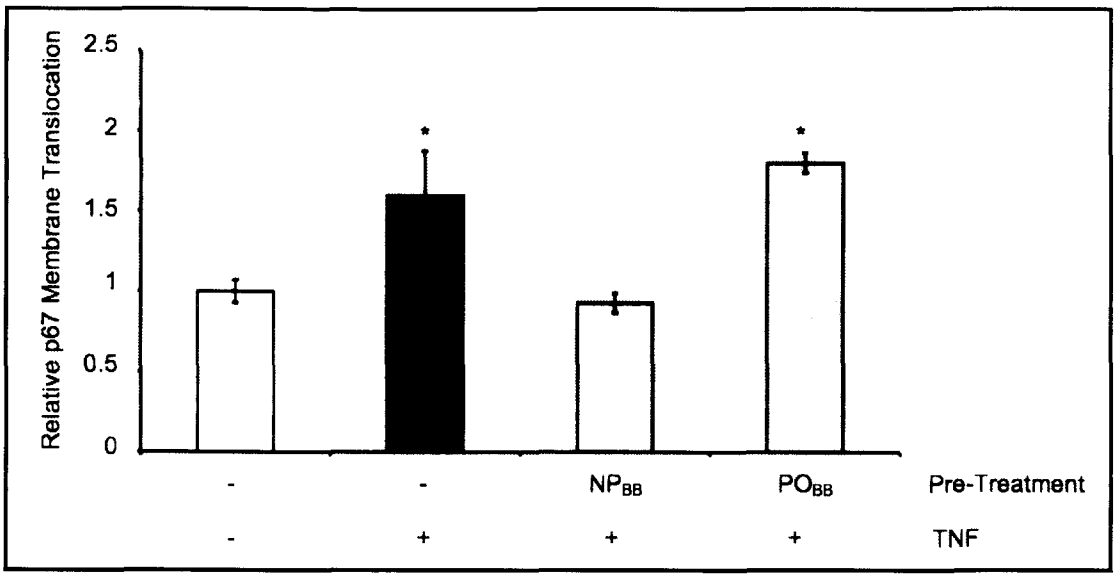

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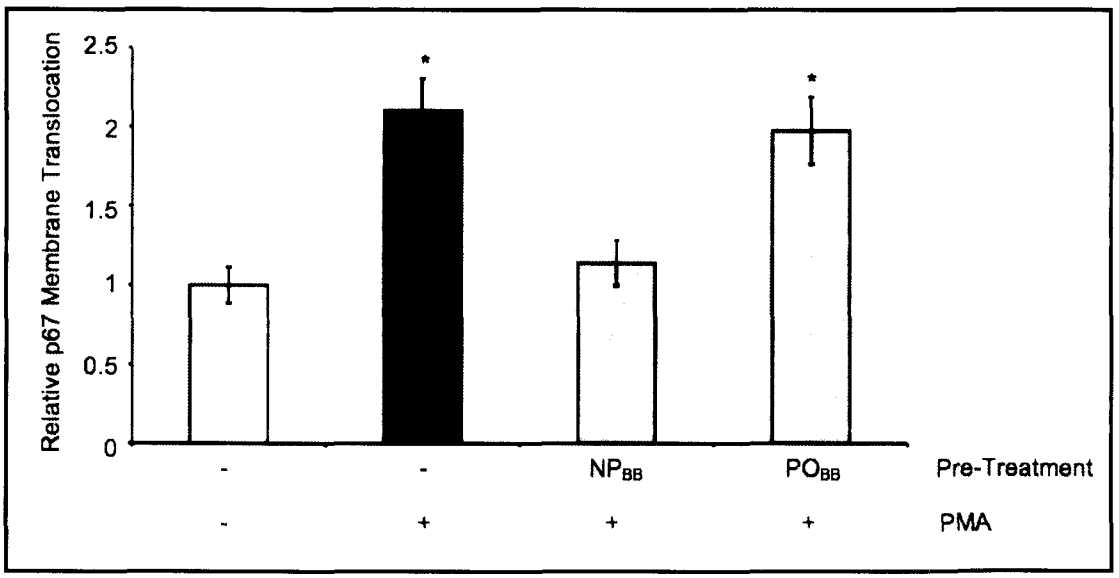

Figure 4.5: Non-polar blueberry fraction abolishes $p67^{phox}$ accumulation in plasma membrane. Serum free cultures of SH-SY5Y were incubated 1 h with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 μg/ml each, 1 h) prior to TNFα (200 ng/ml) or

PMA (400 ng/ml) exposure (30 min). Cells were lysed and fractionated into a cytosolic and membrane fraction. $P67^{phox}$ was detected in the plasma membrane fraction with an ELISA (see methods). (A) TNFα exposure of SH-SY5Y cells caused a significant increase in $p67^{phox}$ in plasma membrane (filled bar) which was negated by the presence of non-polar blueberry fraction (NP_{BB}) compared to control (open bar) whereas a polar blueberry fraction (PO_{BB}) was ineffective. (B) PMA exposure of SH-SY5Y cells also resulted in a significant increase in relative absorbance (filled bar), and was negated by 5 μg/ml of non-polar blueberry fraction (NP_{BB}) compared to control (open bar) but not by a polar blueberry fraction (PO_{BB}). All values were normalized to control, data represent the mean of at least four independent experiments ± standard deviations, and statistical significance was determined at $*p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

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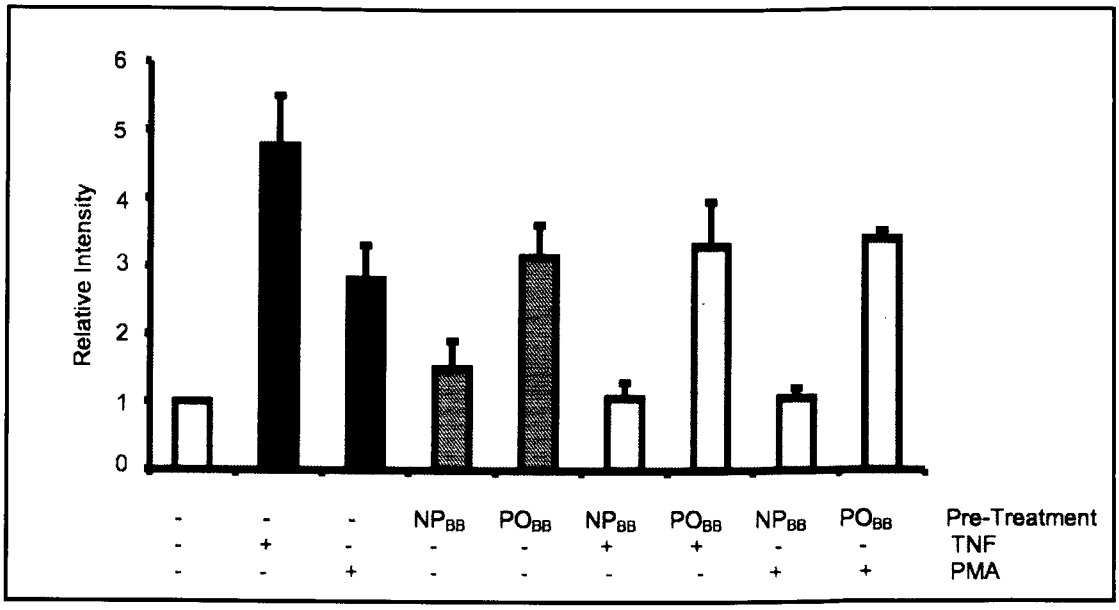

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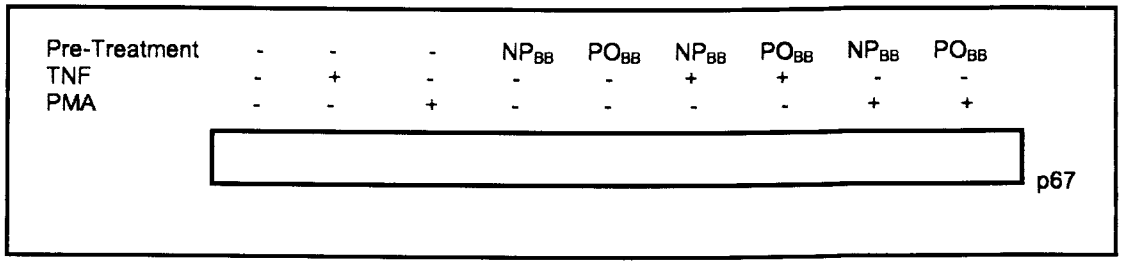

Figure 4.6: Non-polar blueberry fraction inhibits translocation of $p67^{phox}$ into plasma membranes. Serum free cultures of SH-SY5Y cells were incubated with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 μg/ml each) 1 h prior to insult with 200 ng/ml TNFα or 400 ng/ml PMA for 30 min. Cells were lysed and fractionated into a cytosolic and membrane fraction. Equal amounts of total plasma membrane protein were subjected to SDS gel electrophoresis followed by western blotting and detection of immunoreactivity against $p67^{phox}$ (colorimetric detection). Band intensities were

quantified by densitometry (ImageJ64) and all values were normalized to control. TNFα and PMA (filled bars, respectively) induced $p67^{phox}$ translocation, which was abolished in the presence of non-polar blueberry fraction (NP_{BB}) compared to control whereas polar blueberry fraction (PO_{BB}) was ineffective. Note that incubation of SH-SY5Y cells with NP_{BB} or PO_{BB} alone and in the absence of any insult induced a significant $p67^{phox}$ translocation suggesting a small but detectable NOX activation. All data represent the mean of at least four independent experiments ± standard error of the mean (SEM), and statistical significance was determined at $*p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

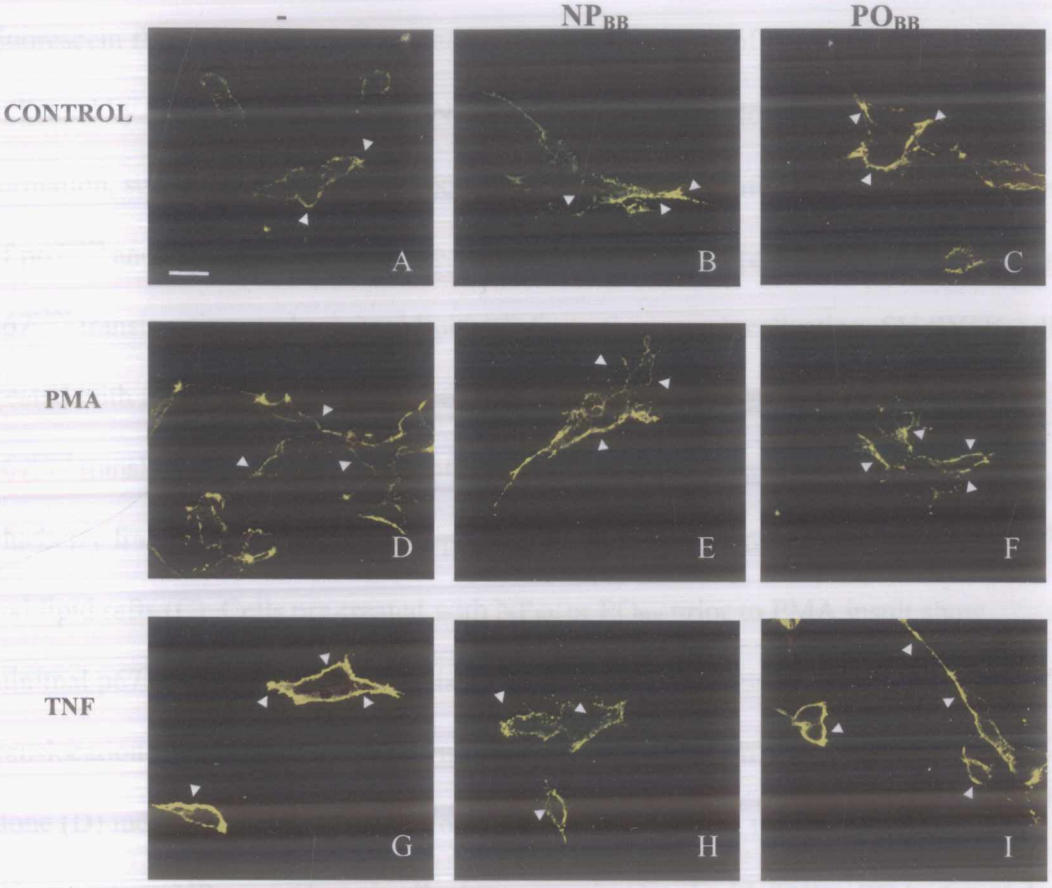

Figure 4.7: Non-polar blueberry fraction inhibits the association of $p67^{phox}$ to the plasma membrane by modulating lipid raft platforms. SH-SY5Y cells were plated and grown on poly-lysine coated glass cover slips until 50% confluent. Cells were serum-starved overnight and then incubated with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 µg/ml each) 1 h prior to insult (200 ng/ml TNFα or 400 ng/ml PMA, respectively) for 30 mins. Cells were then labeled using an Alexa Fluor 555 lipid raft labeling kit, fixed, and permeabalized. Cultures were incubated with anti-$p67^{phox}$ primary antibody and then mounted with PVA-DABCO, and analyzed by confocal microscopy. Images were

acquired (63x, oil, Plan Fluor) under rhodamine fluorescence (lipid raft platforms, red), fluorescein fluorescence ($p67^{phox}$, green), and colocalization of lipid rafts and $p67^{phox}$ is indicated by yellow. As expected, TNFα induced a virtually continuous lipid raft formation, substantial $p67^{phox}$ translocation to the plasma membrane, and colocalization of $p67^{phox}$ and lipid rafts (G) compared to control (A). PMA treated cells (D) show $p67^{phox}$ translocation, and minimal lipid raft formation or colocalization. SH-SY5Y cells treated with non-polar blueberry fraction (NP_{BB}) showed minimal raft formation and $p67^{phox}$ translocation in the absence of insult (B) where as cells treated with polar blueberry fraction (PO_{BB}) did form lipid raft platforms and show colocalization of $p67^{phox}$ and lipid rafts (C). Cells pre-treated with NP_{BB} or PO_{BB} prior to PMA insult show minimal $p67^{phox}$ translocation with discontinuous colocalization puncta (E) and $p67^{phox}$ translocation with some lipid raft formation and colocalization (F), respectively. PMA alone (D) induced $p67^{phox}$ translocation and shows some lipid raft formation and colocalization. NP_{BB} pre-treated cells that were stimulated with TNFα (H) had modulated formations of lipid raft platforms marked by discontinuous colocalized puncta and minimal $p67^{phox}$ translocation compared to the TNFα treated cells (G). Furthermore, TNFα stimulated cells that were pre-treated with PO_{BB} (I) did not show the same modulation effects as cells pre-treated with NP_{BB}. (Scale bar = 20 μm).

Chapter 5

Ursolic Acid Isolated From Lipophilic Blueberry Fraction Inhibits NADPH Oxidase by Lipid Raft Modulation *

5.1 Abstract

The functional assembly of the NOX enzyme in neuronal cells is marked by the translocation of cytosolic factors ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$) to their membrane bound counterparts ($gp91^{phox}$, $p22^{phox}$) and ultimately by the generation of superoxide anion. Membrane bound NOX subunits have been identified in cholesterol rich domains of neuronal cell plasma membranes, otherwise known as lipid raft (LR) platforms (Vilhardt and Van Deurs, 2004). LR platforms provide scaffolding properties for redox signaling that promote translocation of specific NOX proteins and orchestrate the functional assembly of NOX (Klopfenstein et al. 2002). It is documented that lipophilic Alaskan blueberry fractions decrease NOX activity by inhibiting the translocation of $p67^{phox}$ to the plasma membrane. In this study we explore the effects of ursolic acid isolated from a highly lipophilic fraction of wild Alaska bog Blueberries on $p67^{phox}$ translocation. Our findings suggest that the ability of UA to inhibit $p67^{phox}$ translocation is intimately linked to modulation of LR platforms in neuroblastoma cells exposed to tumor necrosis factor alpha (TNFα). The role of NADPH Oxidase (NOX) in neuroinflammation is prominent and contributes to a multitude of acute and chronic central nervous system (CNS) pathologies (Harraz et al. 2008). This investigation confirms NOX as a molecular target

* This manuscript in preparation for submission, Gustafson SJ, Hogan MB, McGill CM, Dunlap KL, Kuhn TB

of neuroinflammation, validates deregulation of NOX by the effects of UA on the plasma membrane of neuronal cells, and further supports nutrition as a critical tool for disease intervention and prevention.

5.2 Introduction

NADPH Oxidase (NOX) is a multimeric enzyme composed of at least two membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$), and three cytosolic subunits ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$). The translocation of the cytosolic subunits to their membrane bound counterparts is phosphorylation dependent and relies on protein-lipid interactions of specific membrane targeting domains (Groemping and Rittinger, 2005; Ueyama et al. 2007). Assembly of NOX, crucial for superoxide formation, a specific type of reactive oxygen species (ROS), occurs in acute immune responses to mediators such as eicosanoids and inflammatory cytokines (Kim et al. 2008). The inflammatory cytokine known as tumor necrosis factor alpha (TNFα) is implicated in oxidative stress associated with various neurodegenerative conditions (Block et al. 2007).

Eukaryotic cell membranes are comprised of several active domains characterized by distinct physical and biological properties. Lipid raft (LR) platform domains are cholesterol and sphingolipid rich region known to provide scaffolding properties and contribute to redox signaling events within the plasma membrane (Vilhardt and Van Deurs, 2004; Klopfenstein et al. 2002; Yang and Rizzo, 2007). The recruitment and assembly of cytosolic NOX proteins are intimately linked to membrane bound NOX subunits that co-localized within LR domains (Eum et al. 2009; Li et al. 2009).

The integration of molecular targets such as NOX into LR domains underscores the importance of LR domains in the quest to reduce oxidative stress associated with neurodegeneration. Compounds such as ursolic acid that are lipophilic in nature and have similar molecular characteristics to cholesterol may have the ability to replace or displace cholesterol in LR domains thus changing the composition of the plasma membrane and intervening with the function of molecular targets. This study aims to examine the effects of ursolic acid, isolated from lipophilic fractions of wild Alaska bog blueberries, on NOX function in relation to LR platforms in the plasma membrane of neuroblastoma cells exposed to TNFα.

5.3 Experimental Procedures

5.3.1 Reagents

Recombinant human tumor necrosis factor alpha (TNFα) was purchased from Millipore (Temecula, CA). DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, TMB, NuPAGE running and transfer buffers, and Alexa Fluor 555 Lipid Raft labeling kit were from Invitrogen (Carlsbad, CA). Fetal bovine serum was received from Atlanta Biologicals (Atlanta, GA). BCA protein assay kit and 1-step NBT/BCIP were from Pierce (Rockford, IL). All other reagents were purchased from Sigma (St. Louis, MO).

5.3.2 Cell Culture

Human SH-SY5Y neuroblastoma cells were grown in 100 mm dishes (falcon) in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (humidified atmosphere, 5% CO_2, 37°C). For amplification, SH-SY5Y cells were treated with trypsin (0.5 mg/ml)/EDTA (0.2mg/ml) for 5 min, rinsed, and immediately resuspended in growth medium onto poly-lysine coated glass cover slips in 35 mm dishes (confocal microscopy) or into tissue culture treated plates. Cells were grown for 24 h to 40% (confocal microscopy) or 80% confluency and then serum starved overnight prior to experimentations.

5.3.3 Cell Viability

Cultures of SH-SY5Y were serum deprived overnight followed by treatments of H_2O_2, and 5, 250, and 500 μg/ml ursolic acid for 48 h. Potential toxic effect on cell viability were monitored using a MTT assay according to manufactures instructions (Millipore) and formazan generation was measured by an absorbance reading at 595 nm with a reference filter of 620 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

5.3.4 Enzyme-linked Immunosorbent Assay (ELISA) for $p67^{phox}$

Cultures of SH-SY5Y cells were grown in 100 mm dishes to confluency and serum deprived overnight. Cells were subjected to 1 h pre-treatments of isolated and pure Ursolic acid (5 μg/ml) prior to exposure of TNFα (200 ng/ml) for 30 min. Cells were

harvested and fractionated into a cytosolic and a plasma membrane pool. Total protein in the membrane fraction was quantified using a BCA assay (Pierce) and 20 ug of total membrane protein was coated per well overnight (96 well high protein absorbent plate, Falcon). Wells were blocked with 5% BSA in 1X TBST (1 h) and then incubated with $p67^{phox}$ primary antibody (3 μg/ml, 4° C, overnight). Next, cells were washed with 1X TBST (3 times, 5 min each), and incubated with corresponding secondary antibody (1:2000, 45 min, RT) conjugated to HRP. Well were rinsed and addition of TMB (100 ul/well, 10 mins) allowed for colorimetric analysis using a Beckman Coulter Multimode DTX 880 microplate reader to measure the absorbance at 620 nm.

5.3.5 Cellular Fractionations

Cells were grown to confluency, treated, harvested and sonicated in sucrose buffer (20 mM Tris-HCL, 2 mM EDTA, 0.5 EGTA, 2 mM AEBSF, 25 μg/ml Leupeptin, 0.33 M Sucrose, pH 8.0). Suspensions were centrifuged (15 min, 16,000 x g) and the supernatant was removed (cytosolic fraction). The pellet was then resuspended and sonicated in a buffer containing 20 mM Tris-HCL, 2 mM EDTA, 0.5 mM EGTA, and 2 mM AEBSF, pH 8.0. Cells were centrifuged (15 min, 16,000 x g) and the supernatant was collected (plasma membrane). A micro BCA protein analysis (pierce) was used according to manufacturers instructions to determine the protein concentration for each sample.

5.3.6 Detection of Lipid Rafts Labeled with Alexa Fluor 555

Cells were grown to confluency, treated, labeled with Alexa Flour 555 (according to manufacturers instructions), harvested in a sucrose buffer and fractionated into cytosolic and membrane fractions as described above. The membrane fraction was then subjected to A/G Agarose beads (100 ul, 30 min, on ice), centrifuged (200 xg_{max}, 2 min) and the supernatant was discarded. The pellet was resuspended in 1% Triton buffer, centrifuged, and the supernatant removed (detergent soluble membrane, DSM). The pellet was resuspended in 1X PBS as the detergent resistant membrane (DRM) fraction and equal amounts of DRM protein for each treatment were transferred to a black 96 well dish (Falcon). Fluorescence detection of Alexa Fluor 555-labeled lipid rafts were measured using a Beckman Coulter Multimode DTX 880 microplate reader.

5.3.7 SDS Polyacrylamide Gel Electrophoresis

Cultures of SH-SY5Y cells were treated, harvested in sucrose buffer, and fractionated into cytosolic and membrane fractions. Total membrane protein concentrations in samples were determined using a BCA protein assay kit. Equal amounts of membrane protein (2 μg) were loaded onto 15% polyacrylamide gels, and subjected to electrophoresis under denaturing conditions.

5.3.8 Western Blotting

Proteins were transferred from Polyacrylamide gels onto nitrocellulose membranes (2.5 h, 125 volts, 50 watts, 250 mA) and membranes were blocked with 5%

w/v BSA in 1X TBST (50 mM TRIS HCl, pH 7.4, 150 mM NaCl, and 0.1% v/v Tween 20). Membranes were probed with primary antibodies (1:500) in TBST overnight, washed with TBST (3 times, 5 minutes each), and incubated with alkaline phosphatase-conjugated secondary antibodies (1:5000 in TBST) for 45 minutes. Immunoreactivity was detected by colorimetric detection using NBT/BCIP (Pierce) according to the manufacture's direction. Band intensities were quantified using ImageJ-64.

5.3.9 Confocal Microscopy

Human SH-SY5Y neuroblastoma cells were grown on poly-D-lysine coated glass cover slips (0.13 mm thick German glass) in medium for 24 h. Cells were serum-starved overnight and incubated 1 h UA (5 μg/ml) prior to acute addition of TNFα (200 ng/ml). Cultures were labeled with Alexa fluor Lipid Raft Labeling kit 555 (Invitrogen) according to manufactures instructions. Cultures were rinsed with 1X TBS and cover slips were mounted with PVA-DABCO. After drying overnight (RT) slides were stored at 4°C until image analysis. Images were acquired (63x, oil, Plan Fluor) with a Zeiss confocal microscope LSM 510 equipped with a He/Ne laser and an Argon laser using 554 excitation and 570 emission filters. Zeiss LSM Software was used for image acquisition and analysis. For each treatment condition, random fields of view were analyzed and 3 cells from two independent sets of experiments ($n = 6$) were examined for the presence of lipid raft platforms (red).

5.3.10 Statistical Analysis

Analysis of variance (ANOVA) was used to determine significance among treatments ($p < 0.05$) followed by Tukey's Post hoc analyses performed using Statistical Analysis System (SAS). All values are expressed as either mean values $\pm$ standard deviations or standard error of the mean.

5.4 Results

The exposure of neuronal cells to TNFα elicits the functional assembly of NADPH Oxidase (NOX) and subsequent formation of superoxide. As previously demonstrated, fractions of a non-polar nature obtained from wild Alaska bog blueberries, inhibited NOX activity by preventing TNFα-mediated translocation of $p67^{phox}$ to the plasma membrane in SH-SY5Y human neuroblastoma cells. Here, we tested whether ursolic acid isolated from non-polar fractions of wild Alaska bog blueberries (IC UA), as well as commercially available UA (pure UA) have the capacity to inhibit $p67^{phox}$ translocation to the plasma membrane of neuronal cells exposed TNFα. Quantification of $p67^{phox}$ translocation was determined by an ELISA for $p67^{phox}$ in the plasma membrane of SH-SY5Y neuroblastoma cells. As shown in Figure 5.1, SH-SY5Y cells subjected to a 30 min exposure of 200 ng/ml TNFα revealed a dramatic increase in $p67^{phox}$ translocation (1.35 ± 0.03, n=4, $^*p<0.05$) compared to control cultures (1.00 ± 0.01, n=4). In contrast, the presence of IC UA and pure UA (5 μg/ml) both negated TNFα-mediated $p67^{phox}$

translocation to the plasma membrane (1.13±0.04, n=4, $p<0.05$ and 1.12±0.03, $p<0.05$, respectively).

Next, we examined the effects of ursolic acid, a compound that is structurally similar to cholesterol, on LR platforms in detergent resistant membranes (DRM) of neuronal cells exposed to TNFα (Fig. 5.2). As expected, SH-SY5Y cells exposed to 10 μM of methyl-β-cyclodextrin (MβCD) for 10 min alone or prior to 30 min exposure to 200 ng/ml TNFα resulted in a significant decrease in LR formation (0.54±0.36, n=6, $p<0.05$ and 0.87±0.15, n=6, $p<0.05$, respectively) compared to cells exposed only to TNFα (1.98±0.32, n=6, $p<0.05$). Cells pre-treated with 10 μM of the neutral Mg^{2+}-dependent sphingomyelinase inhibitor, GW4869 (GW), or with 5 μg/ml of UA for 1 hour prior to TNFα exposure both decreased the presence of LR platforms in DRM (0.60±0.07, n=6, $p<0.05$ and 1.07±0.21, n=6, $p<0.05$, respectively) when compared to control cells (1.00±0.08, n=6, $p<0.05$). Notably, cells exposed to UA in the absence of insult did not alter LR presence in DRM (0.84±0.12, n=6, $p<0.05$) and exposure of SH-SY5Y cells to MβCD post-UA treatment did not significantly impact LR formations (1.28±0.22, n=6, $p<0.05$) when compared to control cells.

Ganglioside-1 (GM1) and flotillin are biomarkers specific to LR domains of the plasma membrane. As shown in Figure 5.3, we examined the effects of UA on gp91 (A), $p67^{phox}$ (B), GM1 (C), and Flotillin (D) in the plasma membrane of neuronal cells exposed to TNFα. As expected TNFα exposure (TNF) increased both GM1 and Flotillin in the membrane of SH-SY5Y cells when compared to cells that were not treated where as MβCD treatments (MBCD and TNF+MβCD) decreased the presence of both GM1 and

flotillin (Fig. 5.3C, 5.3D). For the first time, we reveal that UA decreases TNFα-mediated GM1 and flotillin in the plasma membranes of SH-SY5Y cells exposed to TNFα, suggesting a decrease in LR formation. In addition, we validated a decrease in TNFα-mediated $p67^{phox}$ translocation to the plasma membrane of SH-SY5Y cells pre-treated with UA (Fig. 5.3B). Note that gp91 is a known membrane bound NOX subunit and thus served as an experimental control (Fig. 5.3A).

The viability of human SH-SY5Y neuroblastoma cells upon prolonged exposure to UA was determined to verify the specificity of UA on decreased $p67^{phox}$ translocation and LR detection. Figure 5.4 demonstrates that UA (5 μg/ml) does not compromise cell viability (0.77±0.21, n=8, $p<0.05$) over a 48 h time period compared to control (1.00±0.09, n=8, $p<0.05$) and even at concentrations up to 100 fold (500 μg/ml) higher than the concentration (5 ug/ml) shown to decrease $p67^{phox}$ translocation and LR formations (75 μg/ml: 0.82±0.18, n=8, $p<0.05$; 250 μg/ml: 0.85±0.06, n=8, $p<0.05$; and 500 μg/ml: 0.80±0.14, n=8, $p<0.05$). As expected H_2O_2 (48 h) caused a drastic reduction in cell viability (0.13±0.12, n=8, $p<0.05$, positive control). This result demonstrates that UA does not compromise SH-SY5Y cell viability at concentrations that inhibit $p67^{phox}$ translocation and decreased LR detection.

Lastly, we show the ability of UA to modulate LR platforms in neuroblastoma cells exposed to TNFα using confocal microscopy (Fig. 5.5). Control cells (No Treatment) showed minimal LR presence when compared to cells exposed to 200 ng/ml TNFα for 1 hr. As expected SH-SY5Y cells subjected to MβCD showed a significant

decrease in LR presence while cells treated with 5 μg/ml UA prior to insult with TNFα appear to have modulated LR platforms.

5.5 Discussion

High levels of oxidative stress related to neuroinflammation have been linked to the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), by macrophages of the CNS (Tezel, 2008). TNFα is known to activate magnesium-dependent neutral sphingomyelinase (Mg^{2+}-nSMase) and subsequently liberate ceramide from sphingomyelin in the plasma membrane (Wheeler et al. 2009). In due course, secretion of TNFα orchestrates the progression of neuronal degeneration (Li et al. 2009) through ceramide-dependent cellular stress responses including excessive ROS formation, functional assembly of NADPH Oxidase (NOX), and increased oxidative stress – all of which lead to neurodegeneration. The TNFα-dependent recruitment of TNFα-receptor-1 (TNF-R1) into lipid raft (LR) platforms validates the significant role of TNFα in the formation of LR platforms of plasma membranes (Doan et al. 2004). LR platforms are cholesterol and sphingolipid rich regions of the cellular plasma membrane that provide scaffolding properties for cellular signaling and for the assembly and function of membrane proteins such as NOX.

Our findings reveal for the first time, that ursolic acid isolated from lipophilic fractions of wild Alaska bog blueberries intervenes with the functional assembly NOX in a neuronal model of inflammation. Here, we demonstrate that ursolic acid (UA) isolated from wild Alaska bog blueberries and also commercially available UA both inhibit the

translocation of $p67^{pohx}$ (Fig. 5.1; Fig. 5.3B). Next, we isolated detergent resistant membranes (DRM) from whole cell lysates of SH-SY5Y cells to examine the effects of UA on LR platforms. In this experiment (Fig. 5.2), ganglioside-1 (GM1) was tagged with Alexa Fluor 555 labeled cholera toxin (CTx), crosslinked, isolated, and quantified by fluorescence detection. The structural integrity of LR platforms is compromised when exposed to cholesterol-sequestering agents such as Methyl-β Cyclodextrin (MβCD) (Yang and Rizzo, 2007). The prevalence of cholesterol in LR platforms permitted the use of MβCD as a suitable control for this experiment. Disruption of LR platforms by cholesterol depletion prevents TNFα-dependent recruitment of TNF-R1 to LR platforms (Doan et al. 2004) and thus decreases the presence of GM1 in DRM (Fig. 5.2). Perturbation of LR composition may also alter the function of proteins, such as NOX, that are dependent on LR domains as signaling platforms.

The structural framework that LR domains provide for a variety cellular and molecular signaling creates an ideal environment for the NOX membrane subunits to reside. In fact, the co-localization of $gp91^{phox}$ and $p22^{phox}$ within LR platforms may be crucial for subunit interaction and for the translocation of NOX cytosolic subunits to their membrane bound counterparts. Here, we quantitatively measured $gp91^{phox}$ as a control for plasma membrane protein and show again that UA prevents the translocation of $p67^{phox}$ to the plasma membrane of SH-SY5Y cells exposed to TNFα (Fig. 5.3B). The ganglioside GM1 is abundant in plasma membranes of nerve cells and is known to preferentially partition into LR platforms hence the use of GM1 as a popular LR biomarker. Flotillin, also prominently found in LR platforms of the plasma membrane, serves as an additional

biomarker for LR formation. Using western blot analysis, we quantitatively measured the presence GM1 and flotillin (Fig. 5.3C, 5.3D) in SH-SY5Y cellular membranes treated with UA prior to TNFα exposure. The decrease of GM1 and flotillin in the plasma membrane of SH-SY5Y cells subjected to MβCD demonstrates the ability of MβCD to negate LR platforms. In addition, we show that UA treatments also reduce LR platforms in SH-SY5Y cells exposed to TNFα and that UA is not cytotoxic in this neuronal model of inflammation (Fig. 5.4). Moreover, we demonstrate via cellular imaging that the capacity of UA to inhibit $p67^{phox}$ translocation is interrelated to the modulation of LR platforms in the plasma membranes of SH-SY5Y human neuroblastoma cells exposed to TNFα (Fig. 5.5). In conclusion, we demonstrate UA intervention of LR platforms in plasma membrane using DRM isolation, western blot analysis, and confocal microscopy.

Natural compounds isolated from a variety of plants have shown exemplary health benefits. For example, plant sterols such as beta-sitosterol (Structure 5.1) have been shown to lower cholesterol levels in human studies (Cicero et al. 2002). The bioactivity of natural compounds that comprise wild Alaska bog blueberries are also well documented and are suggested to ameliorate metabolic disorders such as obesity and diabetes (Kellogg et al. 2009). Compounds such beta-sitosterol, and UA (Structure 5.2) isolated from wild Alaska bog blueberries, exhibit structural and dynamic similarities to that of cholesterol (Structure 5.3) and may have the ability to replace or displace cholesterol in the plasma membrane of various cell types. Replacing or displacing cholesterol can compromise the structural integrity of LR platforms and can influence membrane curvature. We speculate that modulation of LR platforms by UA is due to the

structural similarities of UA and cholesterol. Altogether, this research identifies neuronal NOX as a specific molecular target for nutrition-based strategies against inflammation associated with acute and chronic pathologies as well as general aging. Identifying the mechanistic characteristics in which UA modulates LR platforms could provide further insight into the inhibition of NOX-mediated neuronal inflammation.

5.6 Acknowledgements

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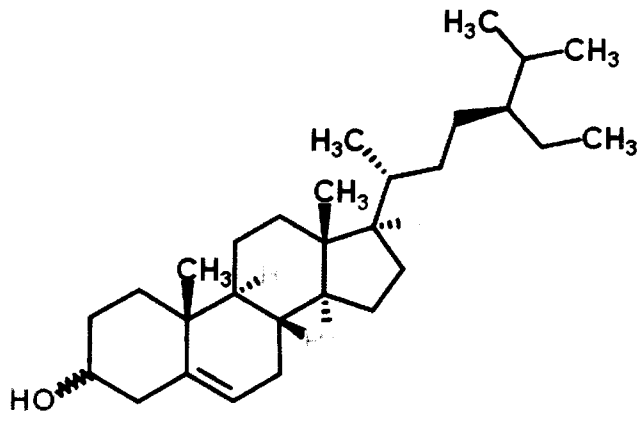
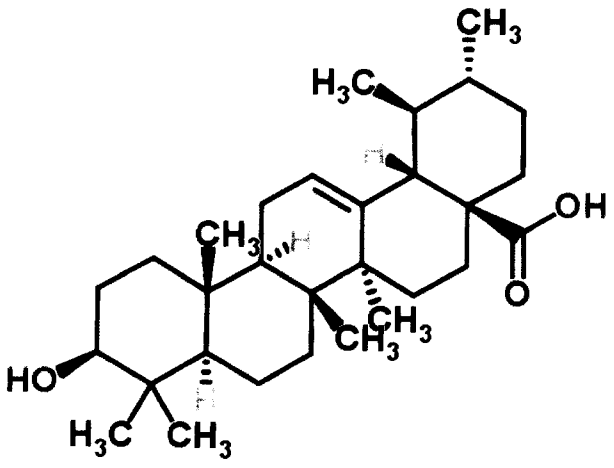
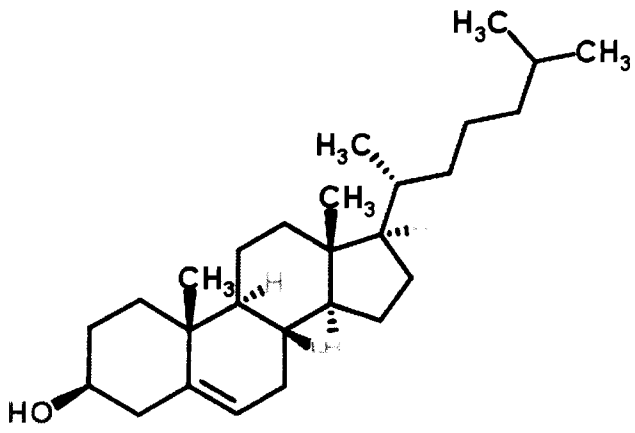


Structure 5.1: Beta-Sitosterol



Structure 5.2: Ursolic Acid



Structure 5.3: Cholesterol

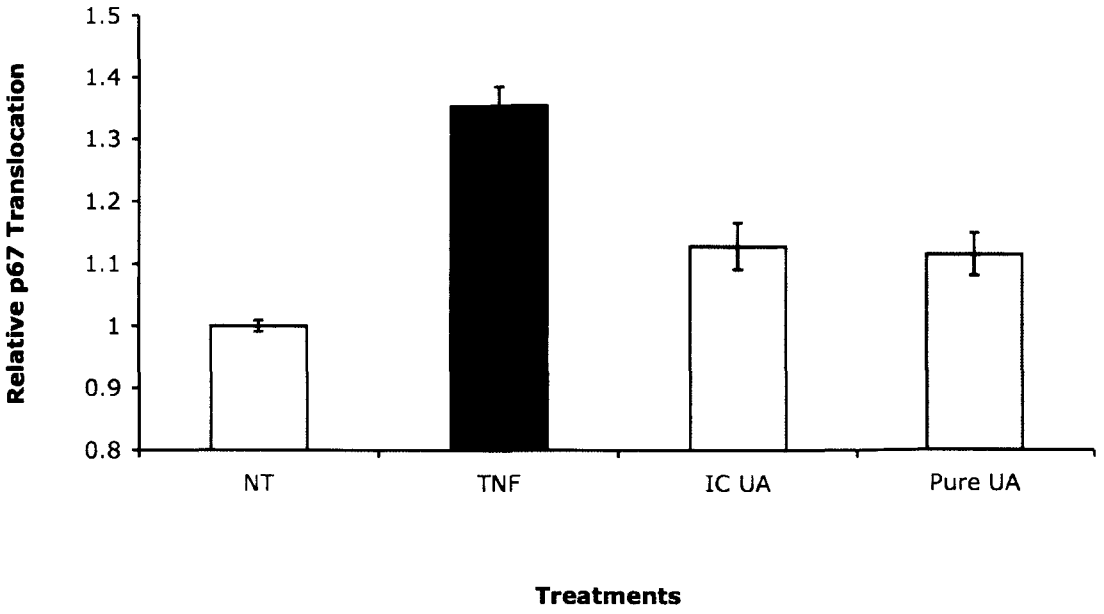


Figure 5.1: Ursolic acid abolishes p67^{phox} accumulation in plasma membrane. Serum free culture of SH-SY5Y cells were incubated with Ursolic acid isolated from Alaskan blueberries (IC UA) and pure Ursolic acid (Pure UA) (5 μg/ml) for 1 h prior to insult with 200 ng/ml TNFα for 30 min. Cells were lysed and fractionated into a cytosolic and membrane fraction. Total membrane protein was absorbed on a 96 well plate (20 μg/ml, overnight). After blocking (5% BSA, 1 h) wells were incubated with rabbit anti-p67^{phox} antibody (1:1000 in 1X TBST, 4° C, overnight) and followed by incubation with goat-anti rabbit secondary antibody conjugated to HRP (1:2000 in 1X TBST, 45 mins, room temp). After addition of TMB (100 ul/well) max absorbance was measured at 620 nm, as an indicator or p67^{pohx} in the plasma membrane fraction. TNFα exposure of SH-SY5Y cells caused a significant increase in p67^{phox} in plasma membrane (filled bar), which was

negated by both, isolated and pure Ursolic acid (IC UA, Pure UA, respectively, grey bars) compared to cells not treated (NT, open bar). Error bars represent standard error of the mean of at least four independent experiments and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

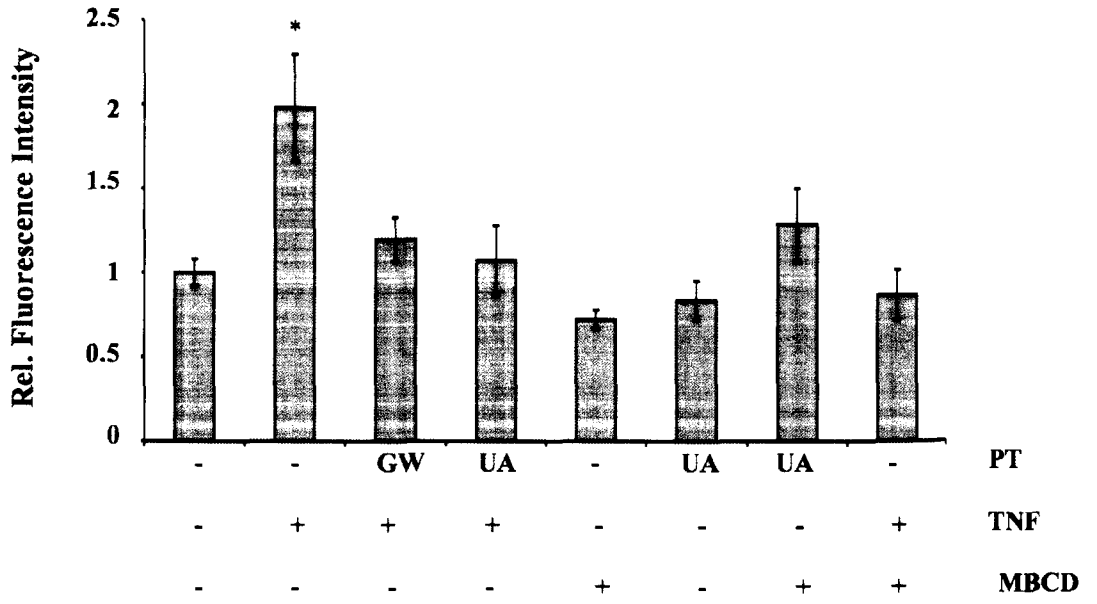


Figure 5.2: Ursolic acid abolishes detection of lipid rafts in SH-SY5Y cells exposed to TNFα. SH-SY5Y cells were plated and grown to 90% confluency. Cells were serum starved overnight and then pre-treated (PT) with Ursolic acid (UA) or GW4869 (GW) prior to exposure of TNFα (200 ng/ml) or MβCD (10 μM). Lipid rafts were labeled with Cholera Toxin-Alexa Fluor 555 (Lipid raft labeling kit, Invitrogen) and then harvested. Lysates were fractionated into membrane and cytosolic fractions and then the membrane fraction was further separated into a detergent soluble membrane (DSM) fraction and a detergent resistance membrane (DRM) fraction. Equal protein from each fraction was analyzed for florescence intensity as an indicator for lipid raft presence. As expected, TNFα exposure increased fluorescence intensity thus indicating an increased presence of lipid rafts in the DRM compared to non-treated cells or cells exposed to MβCD. Cells

exposed to MβCD alone reduced lipid raft presence similar to control in DRM as did cells treated with GW and UA prior to TNFα exposure. Pre-treatment (PT) of cells with UA did not have significant effects on lipid raft presence in DRM when compared with control cells nor did treatments of UA+MBCD or TNFα+MBCD.

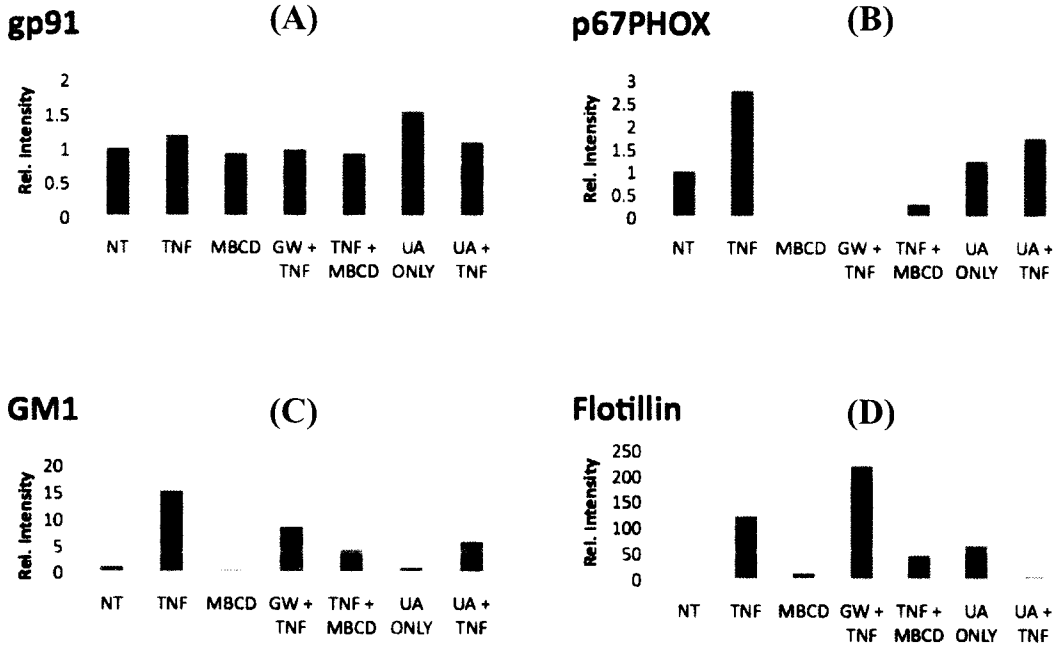


Figure 5.3: TNFα increases p67^{phox}, GM1, and flotillin in the membrane of neuroblastoma cells. Serum free culture of SH-SY5Y cells were incubated with Ursolic acid isolated from Alaskan blueberries (IC UA) and pure Ursolic acid (Pure UA) (5 μg/ml) for 1 h prior to insult with 200 ng/ml TNFα for 30 min. Cells were lysed and fractionated into a cytosolic and membrane fraction. Equal amounts of total plasma membrane protein were subjected to SDS gel electrophoresis followed by western blotting and detection of immunoreactivity against gp91, p67, GM1, and flotillin (colorimetric detection). Band intensities were quantified by densitometry (ImageJ64) and all values were normalized to control. (A) All treatments show similar levels of gp91 detected in the plasma membrane. (B) TNFα increased p67^{pohx} presence (TNF) in the plasma membrane compared to cells not treated (NT). As expected, cells treated with

methyl-β-cyclodextrin (MBCD and TNF+MBCD) or with GW4869 (GW+TNF) negated the translocation of $p67^{phox}$ to the plasma membrane. Cells pre-treated with Ursolic acid (UA+TNF) prior to TNF stimulation show decreased $p67^{phox}$ levels compared to TNFα alone (TNF). Note that Ursolic acid treatments without stimulation (UA only) did not increase $p67^{phox}$ compared to control. (C) Cells treated with TNFα (TNF) increased GM1 in the plasma membrane compared to control cells, cells exposed with methyl-b-cyclodextrin (MBCD or TNF+MBCD), or those treated with GW4869 (GW+TNF). Cells treated with ursolic acid only (UA only) showed minimal GM1 detection while those pre-treated with Ursolic acid prior to TNFα (UA+TNF) show a decrease in GM1 compared to that of cells treated with TNFα (TNF). (D) Cells treated with TNFα (TNF) increased Flotillin detection which was abolished by ursolic acid (UA + TNF) compared to cells not treated (NT). Cells treated with Ursolic acid in the absence of insult (UA only) show minimal Flotillin levels whereas pre-incubation of SH-SY5Y cells with GW4869 (GW) prior to TNFα stimulation induced a significant increase of Flotillin in the plasma membrane.

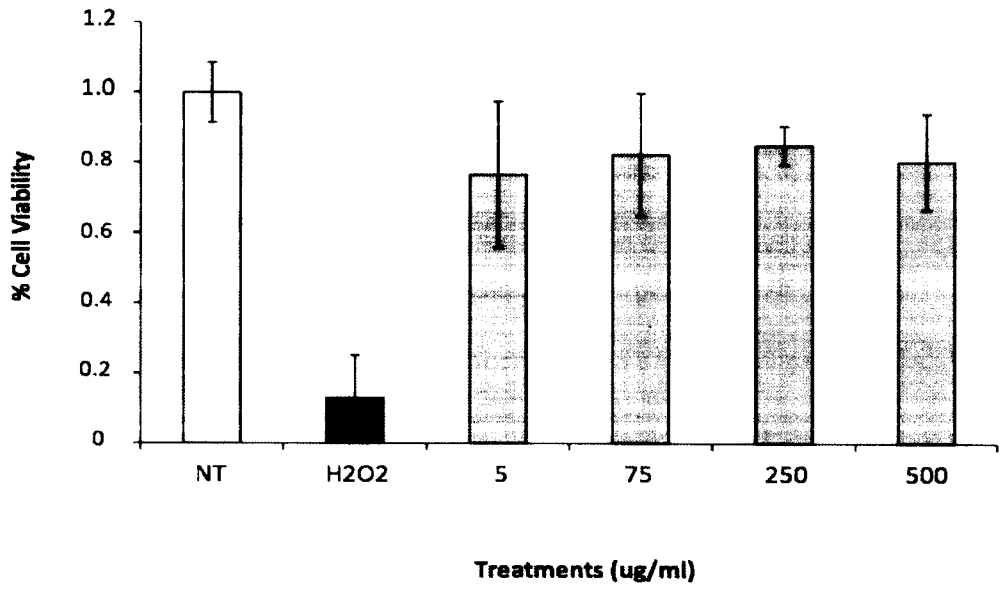


Figure 5.4: Pure ursolic acid is not cytotoxic. Serum free cultures of SH-SY5Y cells were supplemented with Ursolic acid (5, 75, 250, and 500 μg/ml) and maintained for 48 h prior to measuring cell viability (MTT assay). All values were normalized to cells not treated (NT, open bar). Ursolic acid (grey bars) did not compromise cell viability at concentrations (5μg/ml) shown to inhibit $p67^{phox}$ translocation, nor did Ursolic acid compromise cell viability at a 15 (75 μg/ml), 50 (250 μg/ml) or 100 (500 μg/ml) fold excess. As a positive control, H_2O_2 exposure (48 h) of SH-SY5Y cells dramatically reduced cell viability (H_2O_2, filled bar). Error bars represent standard deviations of the mean of at least eight independent experiments and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

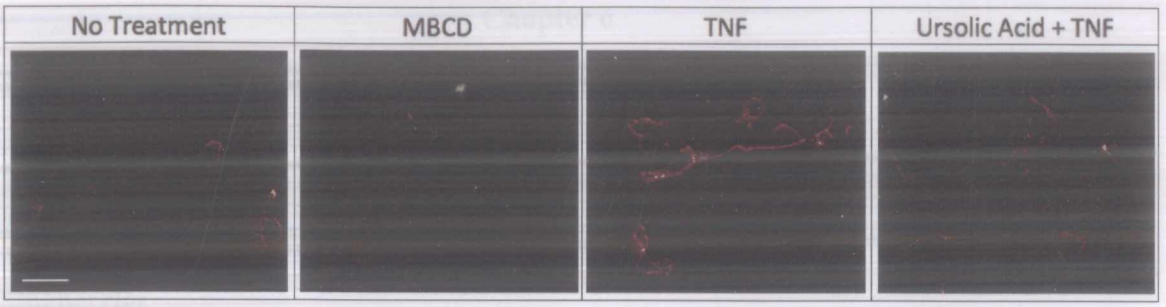


Figure 5.5: Ursolic acid inhibits the translocation of $p67^{phox}$ to the plasma membrane by modulating lipid raft platforms. SH-SY5Y cells were plated and grown on poly-lysine coated glass cover slips until 50% confluent. Cells were serum starved overnight and then incubated with Ursolic acid (5 μg/ml) 1 h prior to insult with TNFα (200 ng/ml) for 30 mins. Cells were then labeled using an Alexa fluor 555-lipid raft labeling kit, fixed, and mounted with PVA-DABCO. Slides were stored at room temperature until imaged using confocal microscopy. Cells with no treatment show minimal lipid raft (red) formation. As expected, methyl-β-cyclodextrin (MBCD) completely abolished any lipid raft platforms present in the plasma membrane of SH-SY5Y cells. Cells treated with TNFα show significant and continuous lipid raft platforms (red) whereas cells pre-treated with Ursolic acid prior to TNFα treatment (Ursolic acid + TNF) show modulated lipid raft platforms in the plasma membrane. (Scale bar = 20 μm).

Chapter 6

Conclusions and Future Directions

6.1 Identification of Natural Compounds Isolated from Wild Alaska Bog Blueberries

Blueberries possess nutritional content, including polyphenolic compounds, that boast antioxidant and anti-inflammatory effects (Piljac-Zegarac et al. 2009). Polyphenolic compounds, including anthocyanins, are interrelated to an increase in neuronal signaling, improved CNS health (Kaur and Ling, 2008), and mediation of memory function (Krikorian et al. 2010). Blueberry supplementation has even been shown to improve memory in older adults (Krikorian et al. 2010). The consideration of wild Alaska bog blueberries having elevated antioxidant capacities is due to the extreme climate in which they cultivate. Alaskan blueberries are exposed to 24-hours of sunlight during the summer months and have hence evolved a thick skin to help them survive the duration of direct sunlight for extensive periods of time. Their thick skin is thought to largely attribute to increased antioxidant content when compared to blueberries grown at lower latitudes or with sun/precipitation balances different than that of Alaska (Kellogg et al. 2009).

While the prosperous effects of antioxidants found in blueberries have been elucidated, less is known about the other naturally occurring compounds that comprise this berry. In these studies we explored the ability of compounds, other than antioxidants, found in wild Alaskan bog blueberries to intervene with a specific biochemical pathway.

In a cell model, we validate that nutrition is tied to defined molecular entities and we demonstrate that blueberries harvested in Alaska have the ability to alter biochemical pathways beyond a passive oxygen radical scavenging capacity. This research illuminates the ability of compounds found in berry fruit to serve as therapeutic agents in the quest of preventing and decreasing neuroinflammation.

Future direction: The isolation and characterization of compounds that comprise the wild Alaska bog blueberry may unveil additional compounds that have ability to intervene with molecular targets of neuroinflammatory pathways. Structural characterization combined with further bio-analysis could provide additional insight into the mechanisms in which these compounds promote neuronal protection. Testing a range of blueberries from various climates and locations could help determine which species of blueberry is most active against neurodegeneration.

6.2 Molecular Targets of Neuroinflammation

High levels of oxidative stress related to neuroinflammation have been linked to the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), by macrophages of the CNS (Tezel, 2008). In due course, secretion of TNFα orchestrates the progression of neuronal degeneration (Li et al. 2009). TNFα is known to activate magnesium-dependent neutral sphingomyelinase (Mg^{2+}-nSMase) and subsequently generate ceramide. Ceramide-dependent cellular stress responses include excessive ROS formation, functional assembly of NOX, and increased oxidative stress – all of which lead to neurodegeneration.

The research presented in this thesis encompasses the effects of wild Alaska bog blueberries on nSMase and NOX as molecular targets known to mediate neuroinflammation. Both of these enzymes are key mediators in a biochemical pathway known to increase oxidative stress and cellular proteome alterations. Our studies show for the first time that crude extracts of wild Alaska bog blueberries, at concentrations without oxygen radical scavenging capacity, have the ability to impede TNFα-stimulated Mg^{2+}-nSMase activity in neuronal cells. We also show that specific fractions of wild Alaska bog blueberry extract have the capacity to inhibit the functional assembly of NOX by preventing $p67^{phox}$ translocation.

Future direction: Uncovering the molecular mechanisms in which natural compounds, isolated from wild Alaska bog blueberries, inhibit nSMase and NOX activity is important for the discovery of novel therapeutic developments. For instance, determining the effects of ursolic acid on the remaining NOX cytosolic subunits could yield a more clear mechanism for NOX inhibition. Discovery of inhibitors for molecular targets, other than nSMase and NOX, can provide further therapeutic options for oxidative stress associated with neuroinflammatory pathways. Examining the inhibitory mechanisms associated with components isolated from wild Alaska bog blueberries is extremely important in combating neuronal degeneration that accompanies acute CNS injuries and chronic CNS pathologies.

6.3 Lipid Raft Modulation

Eukaryotic cell membranes are composed of several active domains that are characterized by their distinct physical and biological properties. Of particular interest are cholesterol and sphingolipid rich regions of the plasma membrane known as lipid raft (LR) platforms. These platforms contain scaffolding properties that intimately link the recruitment and assembly of cytosolic NOX proteins to their membrane bound counterparts (Vilhardt and Van Deurs, 2004). In fact, membrane bound NOX subunits have been identified in LR compartments of neutrophil membranes indicating that LR platforms contribute to redox signaling events that orchestrate the functional assembly of NOX (Klopfenstein et al. 2002).

Methyl-β Cyclodextrin (MβCD) is a cholesterol depletory known to remove cholesterol from LR platforms. When the structural integrity of these domains is perturbed, it causes delocalization of NOX subunits within LR platforms and subsequently decreases ROS levels (Yang and Rizzo, 2007). In this case, NOX and other LR associated proteins redistribute to alternative cellular locations and compromise their standard function (Foster et al. 2003).

For the research presented in the later sections (Chapters 4 and 5) of this thesis, blueberry fractions with the greatest NOX inhibition were honed and determined to be highly lipophilic. These findings lead us to consider that the NOX inhibition presented by lipophilic blueberry fractions could be due to a biophysical effect in the cellular membrane. More specifically, we were interested in the ability of components isolated from this lipophilic fraction to interfere with LR composition. We examined the effects of

Ursolic acid, isolated from a highly lipophilic blueberry fraction, on LR platforms. We can conclude from this research that Ursolic acid effectively inhibits $p67^{phox}$ translocation by modulating LR platforms in the plasma membrane of neuronal cells.

Future direction: Characterizing the effects of various blueberry compounds on the formation and modulation of LR platforms is not only crucial for the study of inflammation of the nervous system but also in the cardiovascular and immune systems. Ursolic acid may replace or displace cholesterol in lipid rafts, which is superior to depleting cholesterol by MβCD due to the cytotoxic effects. As potential therapeutics, compounds other than Ursolic acid, isolated from wild Alaska bog blueberries should be considered for analysis as they too could serve to decrease or prevent conditions associated with NOX activity by modulating or altering the composition of LR platforms. Additionally, the lipophilic nature of inhibitory blueberry fractions increases their probability for crossing the blood-brain-barrier and should be investigated further for treatments designed to improve conditions associated with CNS pathologies.

6.4 Neuronal Models

The experiments presented in this thesis were performed using SH-SY5Y human neuroblastoma cells. The SH-SY5Y neuronal cell line was suitable for modeling biochemical responses to inflammation of the central nervous system (CNS) however translation of our results into primary neurons is essential to correlate the biochemistry presented in this research to relevant physiological outcomes.

Future direction: We have recently begun experimentation with cortical neurons from chick embryos. The plasticity of cortical neurons is vital for organization of neuronal networks as well as overall neuronal function. Loss or degeneration of neuroplasticity, reflected by morphological changes, is evident in inflammatory, acute, and chronic stress models of cell culture and in animal studies. The use of cortical neurons gives us the ability to investigate the morphological characteristics of real neurons exposed to the same experimental conditions presented in this thesis as we aim to establish morphological and plasticity parameters for inflammatory stress responses in the cortical neurons of chick embryos. These experiments may be paired with cortical or hippocampal neurons commercially available from rats or mice for comparative studies. Using cortical neurons as a model for motility, we aim to investigate the benefits of nutritional compounds, isolated from wild Alaska bog blueberries, on dendritic mass and morphology, axon mass and morphology, synaptic density and function, spine architecture and dynamics, and growth cone development. Lastly, we aspire to demonstrate the subcellular localization and regulation of molecular targets, such as NOX and nSMase, and to characterize their associations with lipid raft domains of neuronal plasma membranes.

6.5 References

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